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Abstract: Exosomes are 50-100 nm membranous vesicles actively released by cells which can be indicative of a diseased cell status. They contain various kinds of molecule - proteins, mRNA, miRNA, lipids ¬- that are actively being studied as potential biomarkers. Hereafter I put forward several arguments in favor of the potential use of glycosylphosphatidylinositol-anchored proteins (GPI-APs) as biomarkers especially of cancerous diseases. I will briefly update readers on the exosome field and review various features of GPI-APs, before further discussing the advantages of this class of proteins as potential exosomal biomarkers. I will finish with a few examples of exosomal GPI-APs that have already been demonstrated to be good prognostic markers, as well as innovative approaches developed to quantify these exosomal biomarkers.

Cover Letter

Dear Madam, dear Sir,

please, find a review entitled: 'Exosomes and GPI-anchored proteins: judicious pairs for investigating biomarkers from body fluids'. This review chiefly examines the possibility of using exosomal GPI-anchored proteins regarding investigation of biomarkers. The aim is to highlight common points in proteins sorted in exosomes while drawing lessons from a cell biology standpoint. One main notion considered is that lipid domains behave as sorting devices during exosomes biogenesis.
I believe that this review could be of potential interest to the readership of Advanced drug delivery reviews and more largely to the exosome community, and hope that it will be acceptable for publication.

Looking forward to receiving your decision and thanking you for your consideration,

Best regards,

Michel Vidal

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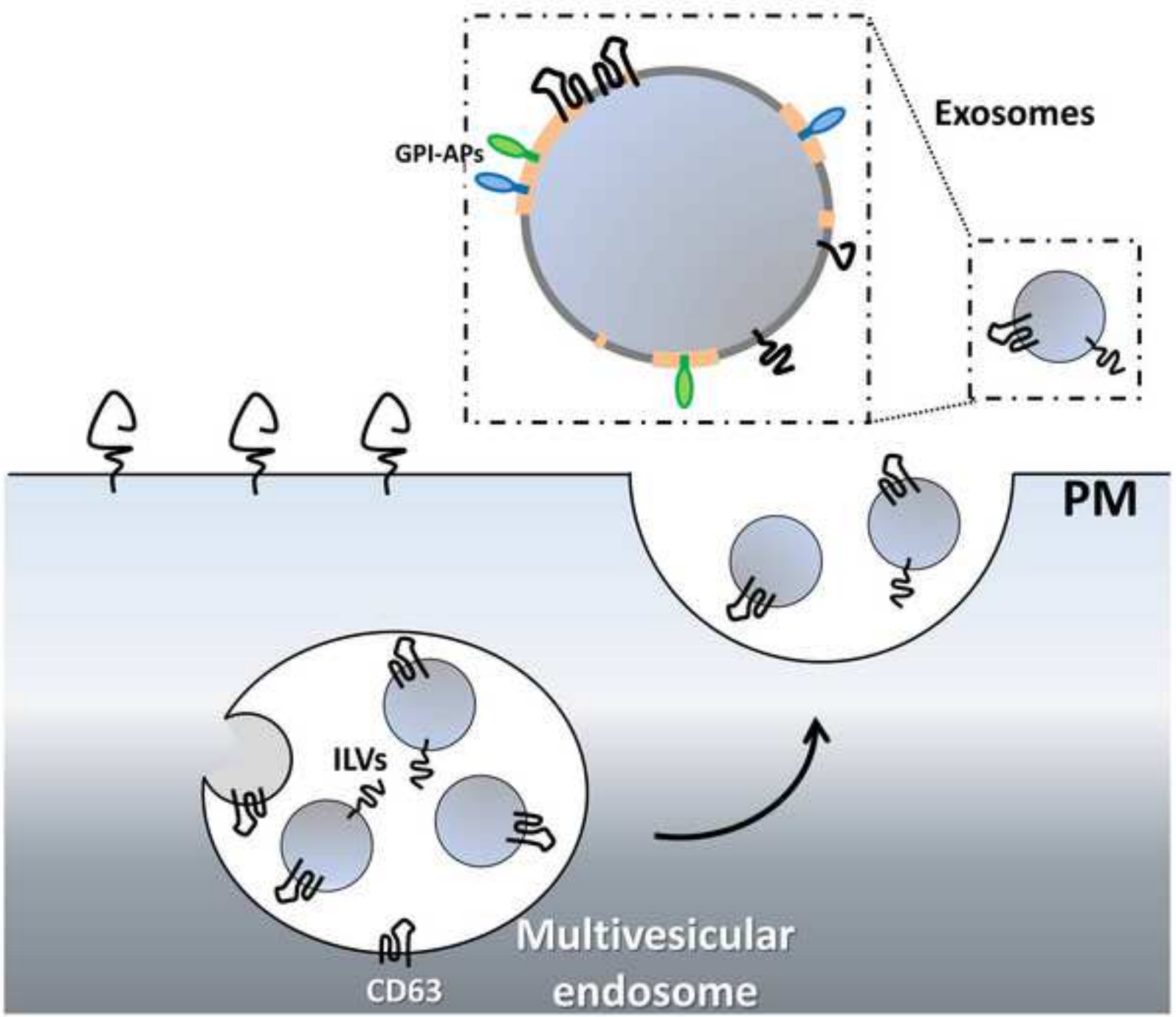
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Exosomes and GPI-anchored proteins: judicious pairs for investigating biomarkers from body fluids

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Abstract
Exosomes are 50-100 nm membranous vesicles actively released by cells which can be indicative of a diseased cell status. They contain various kinds of molecule – proteins, mRNA, miRNA, lipids – that are actively being studied as potential biomarkers. Hereafter I put forward several arguments in favor of the potential use of glycosylphosphatidylinositol-anchored proteins (GPI-APs) as biomarkers especially of cancerous diseases. I will briefly update readers on the exosome field and review various features of GPI-APs, before further discussing the advantages of this class of proteins as potential exosomal biomarkers. I will finish with a few examples of exosomal GPI-APs that have already been demonstrated to be good prognostic markers, as well as innovative approaches developed to quantify these exosomal biomarkers.

1. Introduction

Biomarkers are objective quantifiable characteristics that indicate normal or pathogenic processes. For example, cancer biomarkers can be proteins produced in large amounts by tumor cells, in contrast to healthy counterpart cells. In addition to biopsy tissue sample monitoring, liquid biopsies – urine, blood, saliva, pleural effusions or cerebrospinal fluid – are now very actively investigated. Different types of material – including circulating tumor cells, RNA and cell-free DNA, proteins and vesicles – can be used as biomarkers. Apoptotic bodies, ectosomes and exosomes are three kinds of vesicle – grouped under the term 'extracellular vesicles' – that can be specified according to their biogenesis. Apoptotic bodies are generated by apoptotic cells, whereas viable cells release ectosomes and exosomes. However, exosomes (50-100 nm) derive from an endosomal compartment, while ectosomes/microvesicles (100-1,000 nm size range) are formed through budding of the plasma membrane. They therefore have a distinct membrane composition, with several proteins – including Tsg101, Alix, CD63 – specifying the endosomal origin of exosomes. Moreover, we recently found that ectosomes and exosomes could be used by cells to selectively secrete different parts of cytosolic protein complexes (Figure 1A). This article focuses on exosomes as potential biomarkers, while especially showcasing glycosylphosphatidylinositol-anchored proteins (GPI-APs) as favorable exosomal-associated candidates.

2. Exosomes

2.1. Vesicle biogenesis and membrane protein sorting

Schematically, two types of process are involved in sorting of membrane proteins into intraluminal vesicles (ILVs) of multivesicular endosomes (MVEs). One involves sorting through cytosolic machinery and/or components interacting with cytosolic domains of transmembrane proteins, while a second possibility involves protein clustering in membrane microdomains partly due to their affinity for lipid components. These two processes are not mutually exclusive and could be jointly involved in the biogenesis of the same vesicle (Figure 1B).

2.1.1. Sorting through cytosolic machinery and components

Transmembrane proteins can be finely segregated in endosomes and targeted to lysosomes by a process involving ubiquitin recognition by a cytosolic machinery named endosomal sorting complex required for transport (ESCRT) [1]. Four complexes, called ESCRT-0, -I, -II and -III, are recruited to the surface of endosome on a transitional basis. ESCRT-0, -I and -II take part in transmembrane protein sequestration by binding of ubiquitin coupled on their cytosolic domain. In a second step, ESCRT-III brings about intraluminal budding of the endosomal membrane and is involved in the scission step of the generated ILVs [2, 3]. Exosomes are actually ILVs released in the extracellular space upon fusion of MVE with the plasma membrane instead of lysosomes. Accordingly various ESCRT machinery components or auxiliary components – Tsg101, Alix, syntenin – have been found to be specifically involved in transmembrane protein sorting into exosomes, even without prior ubiquitinylation [4-9]. Note that this sorting is achieved molecule by molecule and requires a whole set of cytosolic proteins.

2.1.2. Membrane related sorting

The membrane lipid part is also involved in exosome biogenesis in an ESCRT-independent way, as demonstrated by secretion of proteolipid proteins (PLPs) in association with exosomes [10]. Inhibition of neutral sphingomyelinase was shown to reduce secretion of PLP in exosomes, pointing out a function of ceramide during the budding process. The membrane lipid component is also involved in protein sorting since definite lipid-anchored proteins and lipids were demonstrated to be sorted in exosomes [11, 12]. Reticulocyte exosomes were used to prepare so-called detergent-resistant membrane (DRM), and proteins such as flotillin, stomatin and Lyn were evidenced in large amount in the prepared fraction [13]. Moreover, tetraspanins constituting a network connected to integrins and MHC class II molecules [14] were shown to be associated with DRM-like structures in exosomes released by B lymphocytes [15]. Note that in this case, the molecular cluster state of proteins – often associated with lipid domains [16] – governs its sorting into exosomes.

2.2. Secretion

2.2.1. Rab protein involvement

Since exosomes are produced in an endosomal compartment, exosomal membrane proteins possibly come from various places – i.e. plasma membrane and Golgi vesicles – before trafficking to the ILV biogenesis site. Consequently, several Rab GTPases could eventually be involved in the protein composition of exosomes since they regulate various transport steps and contribute to vectorial membrane traffic. However, a few Rab proteins have been shown to play a direct and significant role in exosome biogenesis and secretion [17-23]. As anticipated, they are especially involved in endolysosomal vesicle transport towards the plasma membrane. Rab11 and Rab35 regulate membrane components recycling from early endosomes to the plasma membrane while the secretory Rab27 is dedicated to transport of late endosomal/lysosome-like compartments to the

plasma membrane [24]. Rab27-dependence of exosome secretion has been demonstrated in various studies [22, 25, 26].

2.2.2. Environmental influence

In controlled systems such as cell cultures, environmental changes have been shown to modulate exosome secretion in terms of protein composition and the amount of vesicles released. For example, increasing the intracellular Ca^{2+} concentration using various ionophores was long ago shown to enhance exosome secretion in K562 cells [27], a Rab11-dependent process [17]. The effects of such a crude treatment might now be better understood in the light of calcium mobilization during tumor metastasis [28], and also by the fact that Ca^{2+}-stimulated exosome release by cancer cells is a pathway controlled by Munc13-4 [29], i.e. a protein involved in vesicle priming function. Munc13-4 actually uses a Rab11-dependent trafficking pathway to generate MVE competent for exosome release [29].

Another example is the change of protein composition of secreted exosomes when complete medium is substituted by serum-depleted medium. In particular, concomitantly to phosphorylation of eIF2α in producing cells, exosomes secreted in starvation conditions were found to contain translation preinitiation complex components [30]. This could be considered in the light of a recent model based on the assumption that cancer invasion is a conserved adaptation to nutrient limitation [31]. In this model, eIF2α phosphorylation triggered by cell extrinsic signals is positioned as a key event in a 'pseudo-starvation' state, inducing translation reprogramming and mediating phenotypic transitions that drive cancer progression. Exosome secretion might be involved in promoting such translation reprogramming, or at least be an associated event.

Moreover, the tumor microenvironment is known to be acidic, mainly due to the 'Warburg effect' and lactate secretion. Potential benefits of acidosis to cancer cells have been reported, such as decreased susceptibility to hypoxic stress [32] and local immunosuppression [33]. In cell culture, an acidic microenvironment has been demonstrated to promote exosome secretion by various kinds of cancer cells [34-36] and to induce differential expression of proteins [36] and miRNAs [37] in exosomes.

Besides, different studies have demonstrated that there is relationship between exosome secretion and lysosomal pH. Treatment of cell cultures using pharmacological drugs such as chloroquine [38] or bafilomycin A1 [39, 40] has been demonstrated to exaggerate exosome secretion by cells.

2.3. Functional roles of exosomes

Three kinds of functional role of exosomes during their lifetime could be imagined depending on the considered timing, production, transport or capture (Figure 2). The envisaged exosomal function would thus address either producing cells (removal device, disease biomarker) or recipient cells (communication implement).

2.3.1. Exosomes as removal devices

2.3.1.1. Involvement in cellular differentiation

Exosomes were first discovered as a way to get rid of transferrin receptors (TfR) during reticulocyte maturation into erythrocytes [41, 42]. It was then shown that other unwanted or obsolete membrane activities were removed from or decreased on the red blood cell surface by the exosomal pathway [43, 44]. Since reticulocytes – and *a fortiori* erythrocytes – no longer synthesize membrane proteins, exosome release results in definitive loss of the considered proteins. This process leads to remodeling of the red blood cell membrane which involves far more than just the TfR loss [45]. A

critical example is the removal of remnant $\alpha4\beta1$ from the surface of circulating reticulocytes. Integrin $\alpha4\beta1$ is involved in erythroid precursor adherence in erythroblastic islands [16].

Another example of protein removal in relationship with differentiation concerns CD133, which is a pentaspan protein initially described as a surface antigen specific to human hematopoietic stem cells [46]. Hematopoietic stem and progenitor cells (HSPCs) have a capacity for self-renewal and can differentiate into mature blood cells. Moreover, CD133 is now often assumed as a stem cell and cancer stem cell (CSC) marker [47, 48], even though the mechanisms involved in CD133-mediated induction of CSC properties have yet to be completely elucidated. Physiological or experimentally-induced differentiation of HSPCs [49] and colon cancer cell lines [50] respectively, were found to induce secretion of exosomes containing CD133. It is likely that in these cases exosomes serve for CD133 removal concomitantly to its downregulation through transcriptional regulation [51, 52].

2.3.1.2. Involvement in cellular homeostasis

Cells secrete exosomes in response to changes in their environmental conditions or to impairment of degradative compartments, thus enabling them to maintain their homeostasis and integrity (reviewed in [16, 53, 54]). Environmental conditions can drive membrane flux variations, in turn leading to changes in proteins incorporated in exosomal membranes [30]. More interestingly, the cytosolic content can also be modified in the same conditions. In yeast, for example, cell starvation – like oxidative stress – is known to induce disassembly of the 26S proteasome [55] and further separate sequestering of 19S and 20S particles as proteasome storage granules [76]. In mammalian cells, a long period of serum starvation was shown to induce selective release of 19S and 20S proteasomes within exosomes [30] and ectosomes (unpublished results), respectively.

Neurodegenerative diseases are often linked to degradative compartment impairment, leading to toxic protein aggregate accumulation. In Parkinson's disease, cytosolic protein α-synuclein monomers can oligomerize and further aggregate forming β-sheet-rich amyloid fibrils. Exosomes have been shown to play a role in the removal of a toxic oligomeric form of α-synuclein in neuronal cell cultures [56, 57]. The 'hot potato' (exosomes containing α-synuclein oligomers) released by neurons is then supposedly passed to phagocytic microglia for degradation.

2.3.2. Communication implements

2.3.2.1. Phenotype/behavior change

In the light of the finding that B-lymphocytes secrete MHC class II molecule-containing exosomes able to induce antigen-specific MHC class II-restricted T-cell responses [58], it has been suggested that the vesicles have a potential role in immunological processes [59, 60]. More recently, metastatic melanoma cells were shown to release exosomes carrying PD-L1 (programmed death-ligand 1), and in a similar kind of process, i.e. exosome interaction with cell surface receptors, binding of exosomal PD-L1 to its PD-1 receptor on activated T-cells was found to promote inhibition of anti-tumor immunity *in vitro* and *in vivo* [61].

The discovery of mRNAs trapped within exosomes and their translation in recipient cells [62] provided evidence that exosomes are not only able to interact with cells for signaling, but can also transfer their content into cells. Since this breakthrough, a huge number of exosome functional roles have been reported to contribute to various physiologic and pathologic processes [63, 64]. Many of these studies concern the transfer of exosomal miRNAs and their ability to reprogram cell functioning [65]. This is a paramount focus of studies in the exosome field. However, some remaining issues –

such as exactly how the exosome content reaches the cytosol of recipient cells, or the actual physiological impact of exosomes as signaling devices – still require further examination [66, 67].

2.3.2.2. Trophic support

The main fate of exosomes once released in the extracellular space is their capture by recipient cells. Since the majority of published data indicate exosome capture by phagocytosis, endocytosis or macropinocytosis, it could be assumed that biologically active molecules escape from the endolysosomal pathway in order to promote a behavioral change. These captured exosomes might also provide a way to supply cells with nutrients. For example, it was recently shown that exosomes released by cancer-associated fibroblasts supply metabolite cargo that may be utilized by recipient cancer cells after internalization [68]. In the blood circulation, haem molecules contained in reticulocyte exosomes could be recycled for iron homeostasis by splenic and liver macrophages, as is the case for aged erythrocytes [69]. Exosomes can also provide specific constituents to recipient cells. For example, it has been shown that oligodendrocytes secrete exosomes containing PLP, i.e. the major myelin protein [70], and that these exosomes are endocytosed by neurons that retrieve their cargo for neuroprotective functions [20].

2.3.3. Disease biomarkers

All cells secrete exosomes – except mature erythrocytes – and protein composition of exosomes gives an indication of the cell status at any given time. Their membrane composition and/or cytosolic content can differ between healthy and diseased cells. Exosomes are found in various extracellular fluids, thus providing an easily obtained, non-invasive and rapid way for the surveillance of disease progression, as an alternative to tissue biopsy samples. Most studies on exosomes as biomarkers are related to cancer, with specific exosomal proteins identified as being associated with several cancers [61, 71-73], even though miRNAs are increasingly investigated as potential biomarkers [74, 75]. A major problem is to discriminate informative exosomes – secreted by diseased cells – from the bulk of vesicles (including exosomes and ectosomes).

Exosomes could also be used to diagnose infectious diseases since exosomes have been shown to present markers that could be both host and pathogen derived [76].

3. GPI-anchored proteins

Many eukaryotic cell proteins are anchored to membranes by covalent linkage to glycosylphosphatidylinositol. In humans more than 150 proteins are membrane-anchored via a GPI, as highlighted by different technical approaches and membrane fractions [77-79]. Moreover, hundreds of GPI-APs are potentially encoded by the human genome [80, 81]. They perform or mediate various critical cellular functions, including signal transduction, cell adhesion and immune recognition.

3.1. Biosynthesis

GPI are complex glycolipids whose biosynthesis necessitates about 20 distinct gene products involved in the sequential addition of monosaccharides to phosphatidylinositol in the endoplasmic reticulum (ER). A common backbone – comprising an ethanolamine phosphate (EtNP), three mannoses (Man), a glucosamine and phosphatidylinositol group – is synthesized and bulk transferred to proteins in the ER. In mammalian GPI-APs, the first mannose is ubiquitously modified by EtNP (Figure 3). Depending on GPI-APs, the first mannose can also be modified by the addition of glycans.

A detailed description of GPI-AP biosynthesis was recently provided by Kinoshita and Fujita [82]. Very briefly, the first committed step in biosynthesis of GPI anchor occurs on the cytoplasmic leaflet of ER membranes through a complex monoglycosyltransferase comprising seven proteins (including PIG-A). Then, after deacetylation, the obtained glucosamine-phosphatidylinositol is translocated to the luminal face and sequentially processed by ten or so proteins, to yield the mature precursor that serves for protein modification. This mature precursor possesses two side-branch EtNP attached to Man2 and Man3. At this point, the glycolipid backbone is attached to the protein C-terminus by a transamidation reaction catalyzed by a multi-subunit protein complex involving five proteins (including PIG-K). PIG-K is a caspase-like protease that cleaves proteins anchored in the ER membrane through their C-terminus while possessing a GPI attachment signal. The generated C-terminal peptide directs attachment of GPI to the ω site amino acid by transamidation involving the protein GPAA1 [83]. Importantly, during their biosynthesis, GPI-APs are also adjusted on their lipidic part in such a way that the diacyl form of phosphatidylinositol-containing sn2-linked unsaturated fatty acid is modified for a 1-alkyl-2-acyl phosphatidylinositol with an sn2-linked saturated chain.
Interestingly, some genes involved in GPI-AP synthesis were found to be implicated in various cancers. Genes encoding for PIG-U, PIG-T, PIG-S and GPAA1 proteins of the GPI-transamidase complex are overexpressed in almost all cancers, yet not all at the same time in a specific cancer [84]. This suggests that these genes are oncogenes and potential tumor biomarkers. Moreover, this could explain the overexpression and involvement of various GPI-APs in a diverse range of cancers (see below).

3.2. Intracellular traffic and fate of GPI-anchored proteins

3.2.1. Exit from ER

The processes involved in the remodeling of lipidic and glycan parts of GPI-APs during their biosynthesis entail the difference existing between yeast and mammalian cell, regarding GPI-APs export from the ER [85].
In yeast, both lipidic change (acquisition of sn2-linked saturated fatty acid) and glycan modification (removal of one EtNP side-branch) occur in the ER. Consequently, GPI-APs and secretory proteins are sorted in distinct coat protein II (COPII) vesicle populations in yeast [86] because the lipid characteristics of the GPI anchor primarily allow their segregation within lipid domains in the ER membrane. EtNP removal then enables recognition of the GPI-glycan part by the p24 complex, i.e. an adaptor for COPII vesicle sorting (Figure 4A). In contrast, lipid anchor remodeling occurs later in the Golgi in mammalian cells. GPI-APs are thus directly incorporated in COPII vesicles with secretory and transmembrane proteins in mammalian cells.

3.2.2. Trans-Golgi network sorting

As already mentioned, lipid anchor remodeling occurs in the Golgi of mammalian cells and confers new characteristics to GPI-APs. Indeed, after post-translational modifications – including glycosylation – in the various Golgi stacks, GPI-APs are sorted from other transmembrane or secretory proteins at the trans-Golgi network (TGN) level. This process occurs in all mammalian cells but has been studied to a greater extent in polarized cells. Schematically, GPI-APs are preferentially sorted in TGN into vesicles directed to the apical plasma membrane (Figure 4B). The TGN sorting process is known to involve fatty acid saturation of the GPI anchor, which allows association with cholesterol- and sphingolipid-enriched lipid-ordered domains [87, 88]. It is also assumed that protein

oligomerization promotes apical sorting of GPI-APs in TGN, a process that might be favored by different galectins [89-91].

3.2.3. Endocytosis at the plasma membrane

Once at the cell surface – sometimes delivered to the plasma membrane in clustered form – GPI-APs can individually diffuse freely on membrane, but also dynamically partition into lipid nano-domains of a few tenths of nanometers in size. Various studies have been carried out to determine whether such domains exist [92], as first suggested by the findings of experiments involving the purification of detergent-resistant membrane (DRM) fractions [93]. They confirmed that GPI-APs were organized in cholesterol- and sphingolipid-enriched nano-clusters, and required transbilayer acyl chain coupling between the GPI anchor and phosphatidylserine in the inner leaflet [94].

GPI-APs can be endocytosed into specific GPI-AP enriched endosomal compartment (GEEC) vesicles, distinct from clathrin-coated vesicles (CCV) (Figure 4C). This internalization process is regulated by the small GTPase Cdc42 and is sensitive to cholesterol depletion [95]. In BHK cells, GEECs and CCVs are then delivered to a common recycling endosomal compartment. It was demonstrated that recycling from this compartment is slower for GPI-APs compared to other transmembrane receptors, which is due to the GPI anchor and dependent on the cholesterol and sphingolipids levels in the recycling compartment [96, 97]. Interestingly, GPI-APs in CHO cells are transported to late endosomes, suggesting that the association with lipid domains is even stronger and completely inhibits their recycling from the endosomal compartment [98]. In agreement with these findings, the presence of cholesterol [99] and raft-like domains was observed in late endosomes and ILVs [100].

3.2.4. Sorting into ILVs of MVEs

In the processes described above, raft-like domains are involved in GPI-AP sorting within transport vesicles. Here I put forward various arguments in favour of a similar sorting event in exosomes.

Soon after the discovery of exosome release by reticulocytes as a way to segregate and expel TfRs [41], acetylcholinesterase (AChE) activity was observed in the secreted vesicles [43]. Interestingly, the mammalian AChE gene gives rise to multiple alternatively spliced transcripts. There are three main forms, i.e. $AChE_T$, $AChE_H$ and $AChE_R$, where the GPI-anchored $AChE_H$ isoform primarily expressed in erythroid tissues generates dimers. Accordingly, using non-denaturing polyacrylamide gel electrophoresis, reticulocyte exosomal AChE was demonstrated as being sensitive to phosphatidylinositol-specific phospholipase C [12]. Other GPI-APs – CD55, CD58, CD59 – were observed in the same exosomes released by reticulocyte through a method using vesicle immuno-absorption on beads through the TfR and GPI-AP immunodetection by flow cytometry [12]. A previous study showed that AChE clustering using lectins on the reticulocyte cell surface enhanced AChE-associated exosome secretion [11], which is reminiscent of the role of galectins in TGN sorting. Moreover, fluorescent phospholipid analogs were demonstrated to be selectively and efficiently sorted from the endosomal compartment of the same cells after internalization, to rapidly recycle to the plasma membrane (C6-NBD-SM) or accumulate in endosomes (N-Rh-PE) before their release with exosomes [11]. This is also reminiscent of GPI-AP sorting in the endosomal compartment [97, 98], as seen earlier (Figure 4C). Overall, these data indicated a lipid-based sorting mechanism, further supported by biochemical isolation of DRM from exosomes [13]. This raft-like preparation was shown to contain lipid-associated proteins such as flotillin, stomatin, the Src-family kinase Lyn and the ganglioside GM1. Stomatin, flotillin-1 and caveolin are acylated proteins found in oligomeric forms in lipid domains. This is reminiscent of another protein family, i.e. tetraspanin proteins (TSPs), which are

also acylated and interact to form a protein web with affinity to detergent-resistant domains [101]. Certain TSPs were described to interact with MHC class II molecules, while others can associate with integrins [102]. Accordingly, colocalization of MHC class II molecules and TSP in the detergent-resistant membrane domain was observed in B cell-derived exosomes, while MHC class II molecules from the plasma membrane were readily solubilized upon the same treatment [15].

Numerous studies since then have reported the presence of various GPI-APs in exosomes [40, 103-108]. Moreover, expression of anti-epidermal growth factor receptor (EGFR) nanobodies fused to the glycosylphosphatidylinositol (GPI) anchor signal peptide led to the presence of GPI-linked nanobodies on the surface of secreted vesicles [109].

Regarding exosomal lipids, in addition to high glycosphingolipid enrichment [110], it was found that a peculiar phosphatidylserine species (PS 18:0/18:1) was enriched – to a similar extent – together with cholesterol and very-long-chain sphingomyelins. It was suggested that 'hand-shaking' may occur between the long chains of SM and PS respectively associated with the outer and inner leaflets of the vesicle membrane [111]. This is highly reminiscent of transbilayer acyl chain coupling between the GPI anchor and phosphatidylserine in the inner leaflet reported above [94]. Cholesterol is also a critical component of raft domains that are highly represented in exosomes, as highlighted by biochemical [112] and imaging studies [113]. More recently, inhibition of vacuolar-type H^+ -ATPase by bafilomycin A1 was demonstrated to result in a spectacular redistribution of cholesterol via exosome release in HeLa cells [40].

3.2.5. GPI-AP release from cells

Various mechanisms of GPI-AP release from cells – by lipolytic or proteolytic cleavage, release in association with proteins or membrane vesicles – have been described [114].

Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes are secreted by a number of bacteria, cleaving GPI-APs between inositolphosphate and phosphoglycerol residues (Figure 3) and leading to release of a soluble hydrophilic protein. They are mainly used to biochemically characterize GPI-APs [12]. Endogenous PI-PLCs have also been noted in mammalian cells and could contribute to the regulation of their function on the cell surface [115, 116]. A GPI-phospholipase D activity has also been characterized in serum. It cleaves the phosphatidate residue from the inositolglycan-protein moiety (Figure 3). However, it seems that GPI-PLD activity mainly functions on GPI-APs in a solubilized-state rather that in native membrane [117]. On the other hand, certain GPI-APs were reported to be cleaved and released from the membrane by proteolytic cleavage, even though this mechanism – which also occurs with other transmembrane proteins – is not specific to GPI-APs [118].

As seen earlier and discussed below, GPI-APs can be released from cells with their GPI anchor when associated with vesicles such as exosomes or microvesicles. Another possibility involves their association with micelle-like GPI-AP and lipid-harboring extracellular complex (GLEC) structures [114]. These structures differ from exosomes by the absence of lipid bilayer membrane and internal hydrophilic content. Consequently, they can be distinguished from exosomes by the absence of markers such as CD63 or Tsg101. GPI-APs in these GLEC might be the preferential targets of serum PLD.

3.3. GPI-anchored proteins and diseases

There is no doubt about the medical relevance of GPI-APs since specific GPI-APs are critical for tumor growth and invasion, pathogen infection and dissemination. Examples of GPI-AP implication in a few diseases are presented below.

3.3.1. Paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disease that leads especially to hemolytic anemia because of complement regulatory protein deficiency [119]. This is chiefly due to a somatic mutation in the *PIGA* gene whose product is required for GPI anchor synthesis, as part of the complex monoglycosyltransferase referred to earlier. Most of the clinical signs of PNH are caused by a deficiency in two GPI-anchored proteins, namely CD55 (DAF, complement decay-accelerating factor) and CD59 (MIRL, membrane inhibitor of reactive lysis), which normally inhibit the complement membrane attack complex. Exosomes may play a role during the disease [12].

3.3.2. Prion disease

The cellular prion protein (PrP^{c}) is a mammalian GPI-anchored glycoprotein predominantly present on the cell surface in lipid domains. Whether the GPI status is important for misfolding of PrP^{c} to PrP^{Sc} is still a focus of debate, even though the GPI-anchor signal sequence was recently shown to influence PrP^{c} sorting, shedding and signaling [120]. Moreover, cholesterol depletion was shown to interfere with prion propagation [121]. Actually, cells release PrP^{c} – and PrP^{Sc} when infected – in association with exosomes [106], which might be a way by which the disease spreads from cell to cell.

3.3.3. Toxin and virus receptors

Raft domains often represent cellular front doors for bacterial toxins such as botulinum or cholera toxins, respectively binding to GD1a and GM1 gangliosides, or perfringolysin-O insertion in cholesterol-containing membranes. Some of these toxins, e.g. cholera toxin and perfringolysin, have been modified and used to reveal the corresponding lipids in membranes, particularly in exosomes [13, 113]. GPI-APs are also receptors for various bacterial toxins [122, 123] and viruses [124, 125]. Binding of the aerolysin toxin produced by *Aeromonas hydrophyla* requires N-glycosylation of GPI-APs, in contrast to the *Clostridium septicum* α-toxin [126]. Note that the *Clostridium septicum* α-toxin has been used to capture GPI-APs from different biological samples for proteomic analysis [127]. Note also that raft domains can represent a portal of exit for different viruses [128], with GPI-APs being incorporated in viral particles during budding [129].

3.3.4. Cancer and GPI-APs

Many GPI-APs have been found overexpressed in various cancers and are considered as potential biomarkers or targets. Here are just a few examples of the cancer biomarker potential of GPI-APs. Folate receptor (FRα) is a GPI-anchored protein that is preferentially expressed in cancers of epithelial origin – such as breast [130] and ovarian [131] cancers – while rarely being expressed in normal cells. CD109 expression upregulation has been reported in various squamous cell carcinomas [132, 133], while urokinase receptor (uPAR) expression can be used as a prognostic marker in primary and metastatic melanoma cases [134]. GPI-anchored peptidases can be overexpressed in cancers, such as dipeptidase 1 overexpression in colorectal cancer [135], or MMP17 and MMP25 in gastric cancer [136]. Four members of the CEACAM protein family (CEACAM5-8) are associated with the membrane through GPI linkage. CEACAM6 is associated with osteosarcoma metastasis [137], while an antibody-drug conjugate targeting CEACAM5 was used in patients with metastatic colorectal cancer [138]. Glypicans constitutes a 6-member family of heparan sulfate proteoglycans that are all GPI-anchored, with glypican-6 being identified as a putative biomarker for metastatic progression of cutaneous melanoma [139], while glypican-1 has been found to be associated with various cancers

[140, 141]. The prostate stem cell antigen (PSCA) is a cell surface marker that is overexpressed in prostate cancer [142] and is also detected in bladder and pancreas cancers.

4. Exosomal GPI-anchored proteins as biomarkers

4.1. General features

An ideal exosomal protein biomarker should present different features: 1) be over-represented in diseased cells compared to corresponding healthy cells, 2) be efficiently sorted in exosomes, and 3) be exposed on the exosome surface for easier detection.

The GPI-AP family is attractive as a source of potential exosomal biomarkers since a number of GPI-APs match these features. Various GPI-APs have already been found on the surface of exosomes. I will present a few of them that have been demonstrated as potential biomarkers of specific cancers.

4.2. Exosomal glypican-1

As previously mentioned, the six different glypicans (GPC-1-6) constitute a family of heparan sulfate proteoglycans associated with the membrane by a GPI-anchor. The glypican structure includes a core protein, heparan sulfate chains and a glycosylphosphatidylinositol anchor that favors their localization in lipid rafts. The heparan sulfate chains allow interaction with various molecules, such as morphogens, growth factors, chemokines and membrane receptors. Glypican-1 overexpression was initially demonstrated in pancreatic carcinoma cells [143]. Since then, increased GPC-1 expression has been observed in other types of cancer cells [141, 144, 145]. Interestingly, GPC-1 was found enriched in exosomes isolated from cultures of breast and pancreatic cancer cell lines compared to normal counterparts, or from serum of patients with breast or pancreatic adenocarcinoma [72]. The study showed that serum GPC-1 exosomes represented an independent prognostic marker of disease-specific survival. Exosomal GPC-1 detection was then assessed by different methods as a way to detect pancreatic cancer in patients from plasma samples [146-148]. This GPC-1 exosomal feature was also used with patient plasma samples to detect colorectal [149] or prostate [150] cancers, and with urine samples for prostate cancer detection [151].

4.3. Exosomal CEACAM5

Since the initial discovery of the carcinoembryonic antigen (CEA) as a colorectal carcinoma tumor marker [152], other members of the carcinoembryonic antigen-related adhesion molecule (CEACAM) family have been discovered [153]. As already mentioned, four members (CEACAM5-8) are anchored to the membrane through GPI linkage. Note that in the new terminology, CEA corresponds to CEACAM5 while formerly nonspecific cross-reacting antigen (NCA) corresponds to CEACAM6. CEACAM5 and CEACAM6 have been reported as overexpressed in several cancers [154-156]. The LIM1215 colon carcinoma cell line was used to produce exosomes containing Tsg101 and Alix, which were further immunopurified using antibodies against the cell surface A33 antigen [157]. These immunopurified exosomes were shown to contain CEACAM5 identified by mass spectrometry. In another study using the LIM1863 colon carcinoma cell line, exosomes and microvesicles were separately purified by sequential centrifugation to examine their respective protein profiles and potential functions [158]. CEACAM5 was identified in exosomes together with conventional exosome markers – including ESCRT proteins, tetraspanins, Tsg101, Alix and flotillin – although at the same relative abundance compared to the microvesicle fraction. Exosomes from patients' pancreatic duct fluid have also been used to identify protein markers of pancreatic cancer [159]. Pancreatic duct fluid communicates directly with the tumor and was assumed to be a favorable source for collecting exosomes released from pancreatic tumors, while possibly presenting a cancer signature. CEACAM5

was found to be one of the three highly abundant proteins in exosomes collected from pancreatic duct fluid in patients with pancreatic ductal adenocarcinoma [159].

4.4. Exosomal CD24

CD24 is a small, heavily glycosylated protein located in lipid rafts through its GPI anchor. It is a cell adhesion molecule that is reported to interact especially with P-selectin and integrin β1. CD24 is overexpressed in many cancers, including colorectal [160], gastric adenocarcinoma [161], non-small lung [162] and breast [163] cancers. Accordingly, its presence was reported in exosomes collected from various body fluids and types of cancer [164-166]. More recently, molecular profiling of ovarian cancer protein markers was carried out on exosomes by using a nano-plasmonic sensor [167]. First, using exosomes purified from culture supernatant of the CaOV3 ovarian cancer cell line, CD24 (together with EpCAM) was demonstrated to have the highest expression level in exosomes, compared to other selected cancer markers such as CA-125, MUC18 and EGFR. When testing 10 ovarian cancer cell lines, it was found that CD24 and EpCAM constituted a molecular signature of ovarian cancer exosomes compared to exosomes secreted by benign cells. Then ascites of ovarian cancer patients were directly used in the same nano-plasmonic exosome (nPLEX) assay and confirmed the high exosomal levels of CD24 and EpCAM as compared to ascites from noncancer patients. Moreover, the exosomal levels of the two markers were shown to decrease in ascites after chemotherapy of patients responding to treatment in contrast to those of nonresponding patients [167].

4.5. Exosomal melanotransferrin

Melanotransferrin (MTf) is a transferrin homolog that is found predominantly bound to the cell membrane via GPI anchoring [168, 169] (Figure 3). Melanotransferrin was first described as a marker antigen for human melanoma cells [170]. MTf has also been detected in other neoplastic cells and fetal tissues, but only in small quantities in normal tissues [171, 172]. The function of MTf in cells is still not very clear. A role in iron metabolism was first suggested because of its homology with serum transferrin [173], but then it was demonstrated that MTf does not provide Fe to melanoma cells [174]. Various roles of MTf have been reported in relation to cancer progression [175], e.g. in cell invasion [176], melanoma tumorigenesis [177] and angiogenesis [178]. Recently, melanotransferrin was demonstrated to be a serological marker of colorectal cancer on the basis of a secretome analysis and quantitative proteomics [179]. Moreover, it has been shown that melanoma exosomes contribute to pre-metastatic niche settlement [26].

We have provided evidence (unpublished data) that MTf is enriched in exosomes vs lysates of two melanoma cell lines (A-375, SK-MEL-28), to a similar extent in comparison to classical exosome markers, including Alix, Tsg101, syntenin-1 and CD63. Melanotransferrin is found in exosomes secreted by melanoma cells but not by other cancer cell lines, including Caco2, K562, MCF7 and HeLa cells. Melanotransferrin is associated with exosomal membrane via a GPI-anchor, as indicated by its sensitivity to PI-PLC and Triton X-114 partition. We demonstrated that MTf was associated with CD63-positive vesicles which prompted us to set up an assay quantifying exosomal melanotransferrin using anti-CD63 antibody to capture exosomes on the well surface and anti-MTf antibody to quantify melanotransferrin. ELISA quantitation of exosomal MTf is sensitive to detergent and PI-PLC treatment, and allows MTf detection using exosomes at quantities as low as 12 ng protein. Plasma of patients with melanoma vs noncancer patients were tested with this ELISA and the findings confirmed that exosomal MTf could be used as an indicator of the disease. Moreover, exosomal MTf

could be used to obtain pure melanoma-associated exosomes from body fluids for further analysis (e.g. miRNAs).

4.6. Clinical transfer

Even though exosomal markers cannot yet be considered as clinical diagnostic markers, there is no doubt about their prognostic potential. Exosomal biomarkers can provide useful indications for monitoring patients. The treatment efficacy in patients can be relatively quickly determined based on variations in the rates (e.g. decrease/normalization of the biomarker when the treatment has succeeded in eliminating the cancer cells)[167].

Direct detection using plasma [148], pancreatic fluids [159], lymph exudates [180, 181], urine [151], amniotic [164] or cerebrospinal fluids [182] increases the possibility of observing disease-specific exosomes. Moreover, new innovative technical approaches to detect and quantitate proteins on exosomes have been developed in recent years which could help find new exosomal biomarkers for clinical use. Three examples are presented below.

4.6.1. ExoScreen

ExoScreen [183] is a system, based on AlphaLisa technology, to detect the co-presence of two specific proteins on the surface of exosomes by a bead-based amplified luminescent proximity homogenous assay. Upon laser excitation, a photosensitizer-containing donor bead converts oxygen to an excited singlet state diffusing across the sample, which potentially generates luminescence from a neighboring (less than 200 nm) acceptor bead. The donor bead is associated with exosomes by antibodies against a classical exosome marker (e.g. CD63), while the acceptor bead is combined with antibodies against the potential biomarker. Using this assay, CD147 was determined as an exosomal biomarker of colorectal cancer by comparing sera from healthy donors to those of patients. Moreover, exosomal CD147 levels decreased after surgery, thus strengthening the status of CD147 as biomarker of the disease [183].

4.6.2. Nano-plasmonic sensor

Surface plasmons are very sensitive to the properties of the materials on which they propagate. The sensing mechanism is based on the detection of local refractive index changes due to the binding of exosomes on a metal film patterned with periodic nanohole arrays functionalized with specific antibodies. Spectral shifts or intensity changes are proportional to the target marker protein levels bound on the chip, or so-called nano-plasmonic exosome (nPLEX) sensor [167]. Using CD63 as target molecule, the authors established that the detection limit of the nPLEX assay was around 3,000 exosomes, i.e. a 100-fold higher sensitivity than that of chemiluminescence ELISA. As seen earlier, this technical approach revealed that CD24 constitutes a molecular signature of ovarian cancer exosomes [167]. This technique may be used for high throughput analysis and is reusable for a further round of measurement, while bound vesicles can be released to analyze their content (e.g. miRNAs).

4.6.3. ExoView® platform

The single particle interferometric reflectance imaging sensor (SP-IRIS) allows phenotyping and digital counting of exosomes. A polymer coating functionalized with antibodies against exosomal proteins enables vesicle capture on a microarray-based solid-phase chip. Individual exosomes are imaged based on interferometric digital sensing, allowing assessment of the exosome concentration, relative size distribution and phenotyping in biological fluids [184]. This approach was developed in the

ExoView® platform using a multiplexed microarray chip for immune-capture of vesicles through tetraspanins surface expression [185]. Fluorescence imaging of potential biomarkers – up to 4 markers on a single vesicle – can be achieved. Colocalization of markers (as a percentage of the population) can be carried out.

5. Conclusion

Here I have put forward several points advocating exosomal GPI-APs as interesting biomarkers, particularly of cancer diseases. GPI-APs are especially attractive because of their potential for efficient sorting in exosomes. Moreover, they are accessible at the exosomal surface and often overexpressed in various cancers. They are thus perfectly for use with detection methods such as those mentioned above. They could improve the prognosis of specific diseases when used together with other exosomal surface biomarkers. Finally, as mentioned earlier, dozens of GPI-APs are yet to be discovered [80, 81] and exosomes might be a favorable basis for such studies.

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Highlights

- Exosomes are extracellular vesicles (80-100nm) derived from an endosomal compartment
- GPI-anchored proteins are overexpressed in various cancers and can often be used as biomarkers
- GPI-anchored proteins are efficiently sorted in exosomes and are present in meaningful amounts
- GPI-anchored proteins are exposed on the surface of exosomes and thus easily quantified

Figure legends

Figure 1: **Ectosome *vs.* exosome biogenesis.**

A - Budding of the limiting membrane toward the endosomal lumen generates intraluminal vesicles (ILVs) within multivesicular endosomes (MVEs). MVEs then fuse with the plasma membrane and release exosomes into the extracellular space. In contrast, ectosomes (or microvesicles) are formed by direct outward budding of the plasma membrane. Besides the different biogenesis features, the two kinds of extracellular vesicles can be used by cells to selectively export parts of the same complex, as illustrated here with proteasome ([30] and unpublished).
B - Schematic representation of protein sorting involved in exosome biogenesis. For the sake of clarity, I deliberately chose to represent cargo sorting possibilities through only two main processes involving ESCRT and associated actors (blue framed), or lipid domains and associated components (yellow framed).

Figure 2: **Functional roles of exosomes.**

During the exosome lifetime, three kinds of functional role for them can be considered (colored frames). They schematically correspond to production, transport or capture events and address either producing cells (green and purple frames) or recipient cells (red frame).

Figure 3: **Structure of GPI-anchored proteins and MTf.**

A common backbone is illustrated here with an ethanolamine phosphate (EtNP), three mannoses (green circles), one glucosamine (pink square) and phosphatidylinositol (yellow hexagon for inositol), and it is conjugated to proteins. Selective cleavages of phosphatidylinositol by PI-PLC and PI-PLD are indicated. Note that melanotransferrin binds iron only through its N-terminal domain.

Figure 4: **Sorting of GPI-APs in various compartments.**

A - *Sorting in yeast ER*. GPI-APs exit the ER separately from transmembrane proteins. GPI-APs are first clustered in lipid domains (highlighted in orange) before sorting into vesicles through COPII (blue symbols) recruitment.

B - *Sorting in TGN of polarized cells*. GPI-APs are sorted in vesicles with lipid domains (highlighted in orange) and mainly directed to the apical side, while other vesicles are routed to the basolateral side of polarized cells.

C - *Internalization and fate of GPI-APs*. GPI-APs can be found in nano-lipid domains on the cell surface and further internalized in so-called GEEC vesicles. After fusion with the endosomal compartment, they seem to be experiencing difficulty in recycling and can be sorted into ILVs of MVE.

Figure 1A

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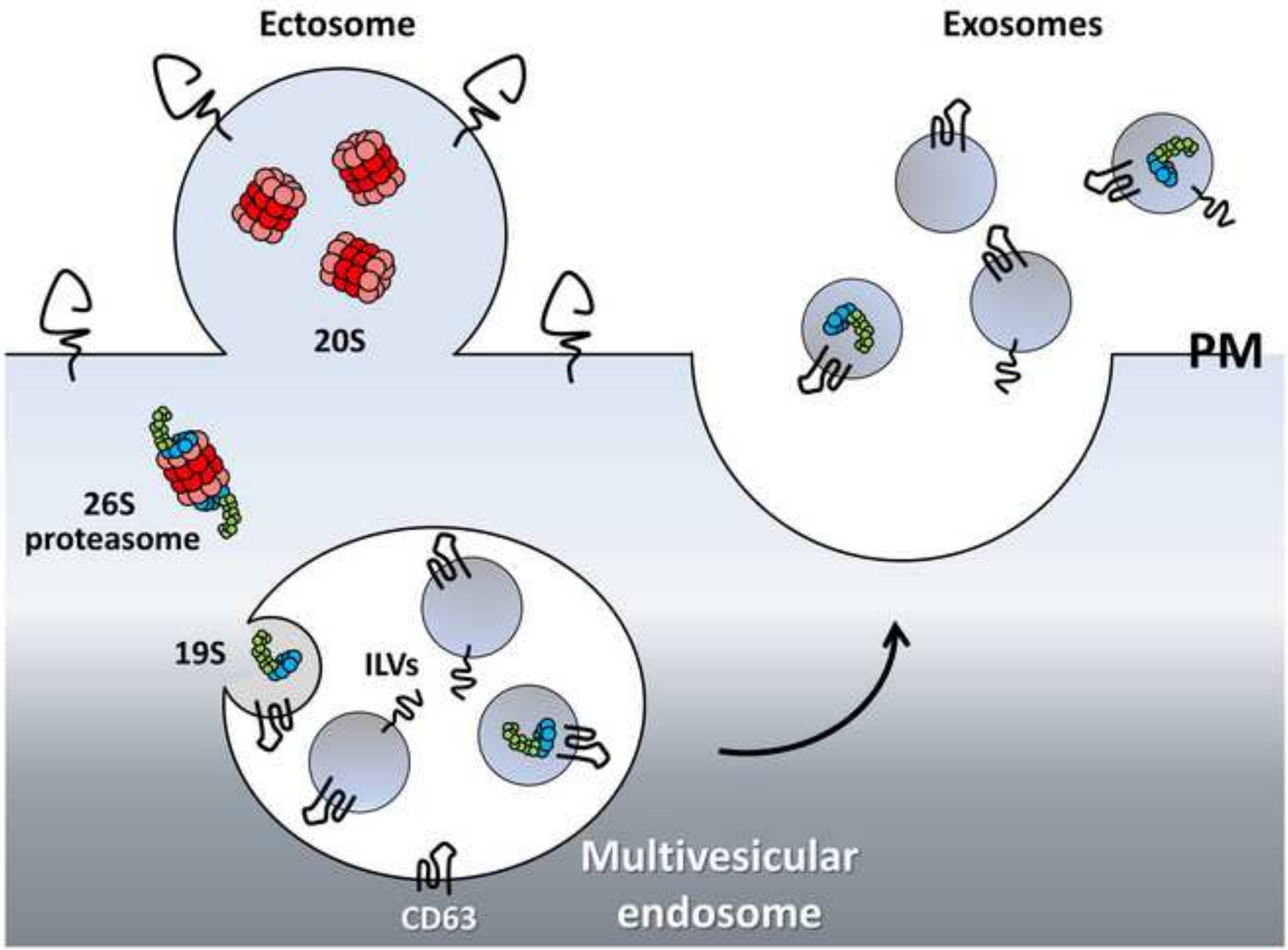


Figure 1B

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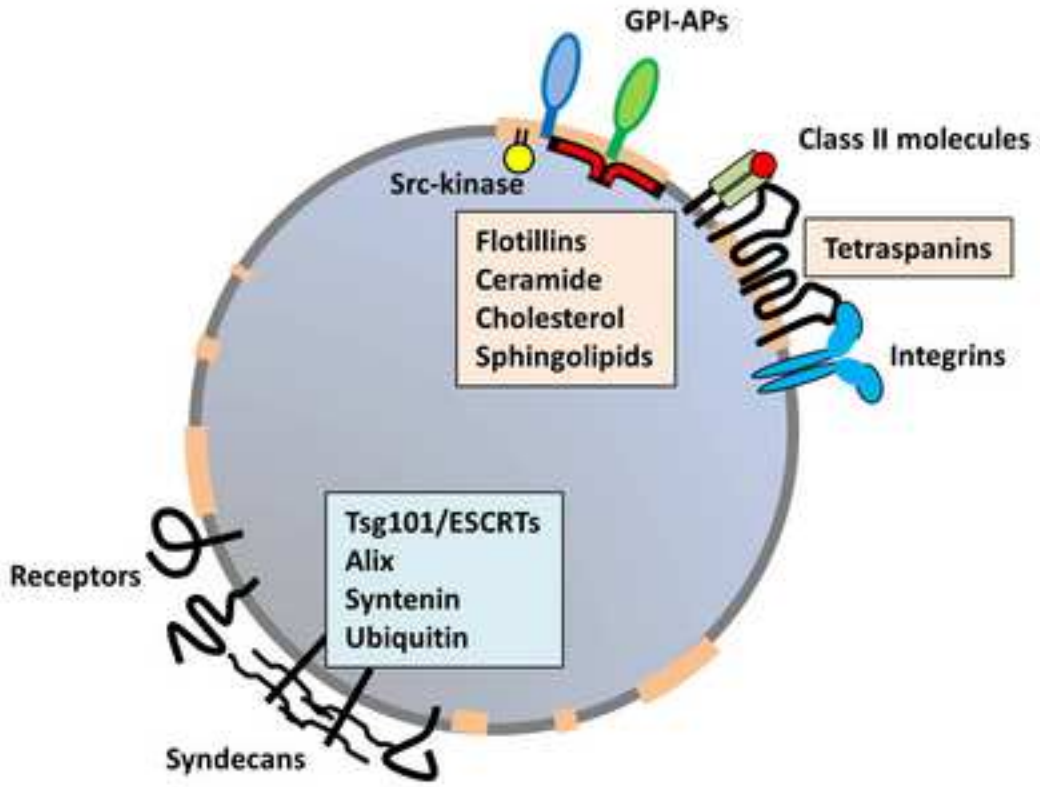


Figure 2

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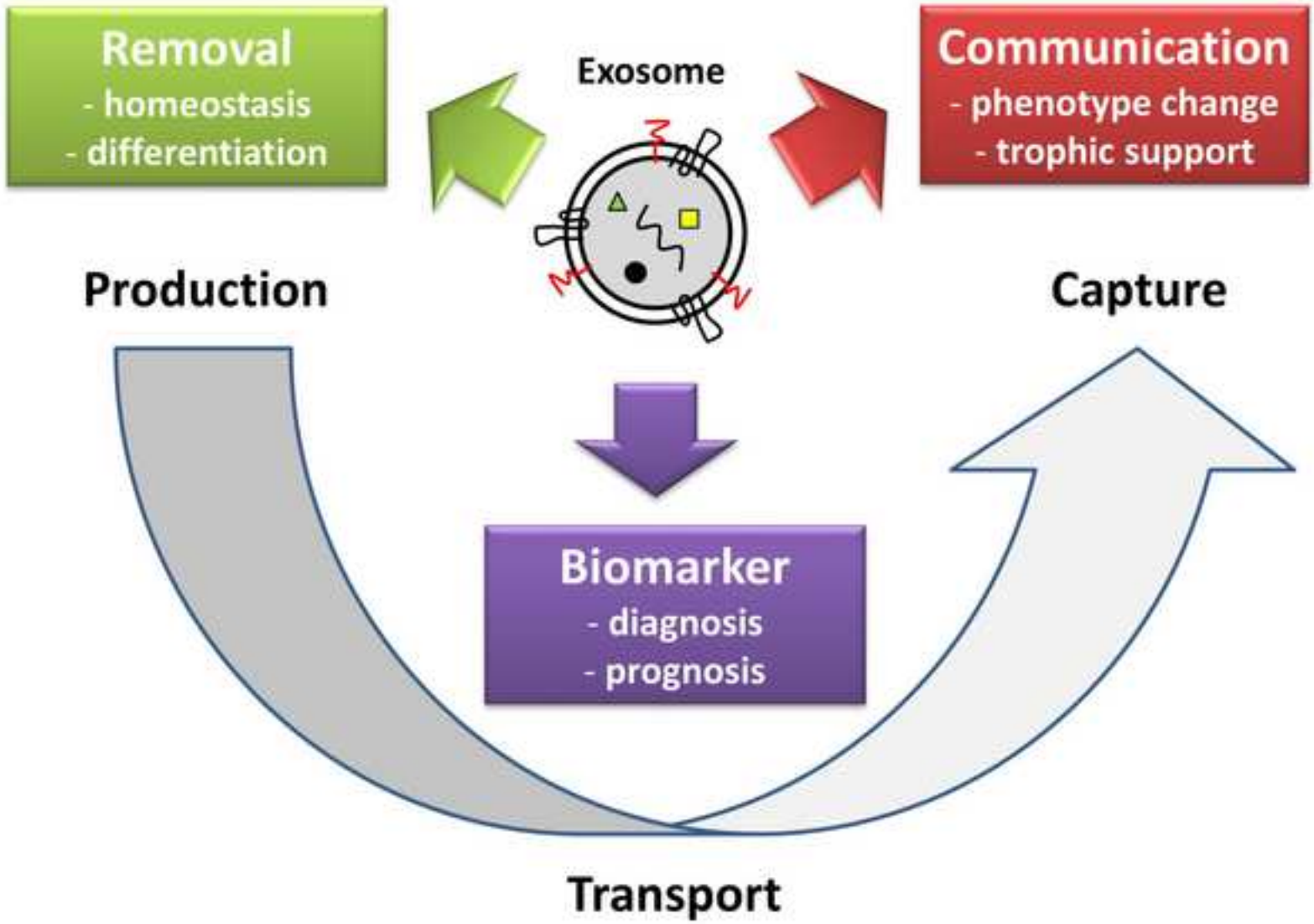


Figure 3

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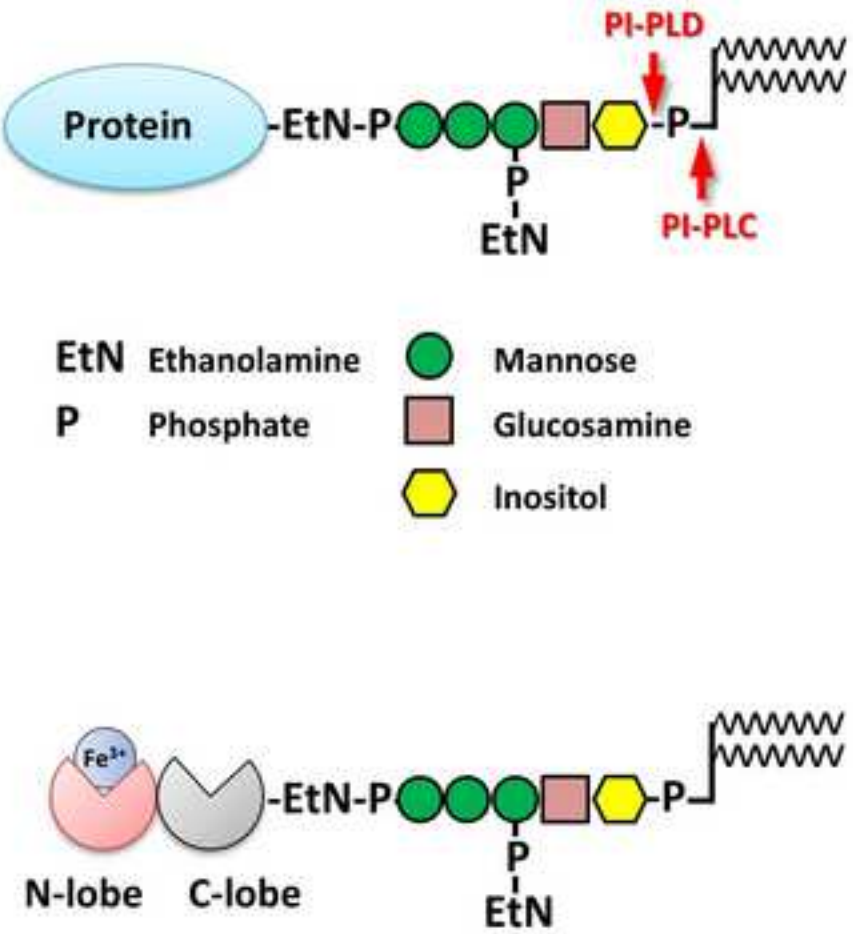


Figure 3

Figure 4

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