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EXOSOMES AS BIOMARKERS AND THEIR PREDICTIVE VALUE

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Exosomes are extracellular vesicles derived from the cell membrane, first reported a few decades ago and they have been the center of interest of researchers due to their various functions, their capacity to mediate intercellular communication and to provide information about the disease status. Exosomes contain a wide variety of molecules, including proteins, RNAs, lipids, and fragments of genomic DNA and their cargo can express the physiological state of the cells they originate from[53]. Studies report that different exosome- derived proteins can serve as biomarkers for the isolation and quantification of exosomes, thus distinguishing them from other vesicles[10,43,44]. All cell types secrete exosomes and data suggests that exosomes released from metabolically active cells can initiate metabolic reprogramming in the end target organs[29]. These observations emphasize the idea that the domain of exosomes can be a potential platform for the progression of metabolic disease. Their non-synthetic profile and higher immuune tolerance of the human cells turned them into possible drug vectors offering an impressive improvement in the farmaceutic domain[5].

Keywords: exosomes, intercellular communications, biomarkers, drug vectors.

INTRODUCTION

Exosomes are extracellular vesicles derived from cell membranes, first reported a few decades ago.[13,21] Their capacity to mediate intercellular communication[36,40] and transmission of disease states, combined with their non-synthetic profile and greater tolerability turned them into possible drug vectors[7,57]. Recent reports of clinical trials assessing exosome-based oncologic therapies claim benefits over conventional treatments, such as usage of lower doses and decreased rates of immune reactions. Although exosomes show great potential as therapeutics, many of their characteristics are still unknown.

EXOSOMES

EXOSOMES DISCOVERY

"Exosomes" belong to a large family of membrane vesicles named extracellular vesicles, secreted by a majority of cells.[64] Exosomes were discovered in 1983 by Harding and Pan[14,18] and were initially considered to be "debris" generated by the cells. In order to investigate the passage of transferrin receptors from plasma membranes into the reticulocytes, the researchers grew reticulocytes with labeled transferrin receptors[21,22]. They discovered that the labeled transferrin receptors were internalized and then packaged into vesicles (~50 nm) inside the reticulocytes. Subsequently, these vesicles were secreted out of the maturing blood reticulocytes into the extracellular space.[25,48] There are three major vesicle populations: apoptotic bodies, microvesicles (MV or ectosomes) and exosomes.[5,24] Their classification depends on their origin, their morphology and mode of secretion into the extracellular space.

CHARACTERIZATION OF EXOSOMES

Exosomes are nano-sized vesicles (50–120 nm in diameter) originating in multivesicular bodies (MVB). They have a lipid bilayer with an average thickness of ~5 nm. This lipidic bilayer includes ceramide, cholesterol, phosphoglycerides and

saturated fatty-acyl chains. Its outer surface is rich in saccharide chains, such as mannose, alpha-2,6 sialic acid, and N-linked glycans[2,12]. Exosomes have a cup-shaped morphology, when examined under an electron microscope[67]. They appear as flattened spheres of 30 to 150 nm diameter[27,28,31]. The release of exosomes into the extracellular medium is mediated by Rab GTPases and occurs by the fusion of the matured MVB with the plasma membrane. The endosomal sorting complex required for the transport (ESCRT) pathway facilitates membrane remodeling and has been involved in the formation of exosomes.

MVBs can be produced in the absence of the ESCRT complex subunits so there is also an ESCRT-independent pathway. Stuffers *et al.* (2009) reported that exosomes can be generated through ESCRTs-independent routes, therefore other mechanisms are also plausible, though less known. Possible explanations came from Edgar et al (2014) mentioning the implication of tetraspanin CD63, while Trajkovic *et al.*(2008) supported the bending properties of ceramides. Immune cells such as B lymphocytes, dendritic cells or mastocytes are able to release exosomes when stimulated by cellular signals. Buschow *et al.* (2009) observed that murinic dendritic cells, which are specialized to activate T lymphocytes, secrete higher levels of exosomes after interaction with antigen-specific CD4+ T lymphocytes. Furthermore, Blanchard *et al.*(2002) and Muntasell *et al.* (2007) found data that T cells and B cells interactions with immune cells lead to higher level of exosome secretion. The best way to define exosomes biochemically is to identify specific markers. Data suggests that cargos (such as MHC II from B cells) and other cell type-specific antigens may help to differentiate exosomes from other extracellular vesicles. These markers can be proteins that are specific to the endosomal pathway, for example proteins related to MVB biogenesis (such as Tsg101, Alix), tetraspanins (CD-63, CD-9, and CD-81), membrane fusion proteins (RAB GTPases and Annexins), signaling molecules (cell adhesion molecules, growth factor receptors) and heat shock proteins (HSP-70 and HSP-90). [11]

EXOSOME ISOLATION TECHNIQUES

Resorting to conventional methods such as fluorescence or electronic microscopy to characterize exosomes is difficult, given their size.[9,18,23] Théry *et al.* (2006), described various approaches for exosome purification, from differential centrifugation from culture medium to the more common pooling from cellular supernatant or animal fluids. Lötvall et al, 2014, stresses on the necessity to include a minimal set of requirements in exosomes characterization[13,31], a field that has recently began to raise deeper interest amongst researchers. Exosome isolation is an area of research that has impressively progressed over the past decade. Exosomes have been isolated from complex biological matrices such as blood, urine, and cerebrospinal fluid.[60,61,74] We will review four groups of exosome isolation techniques: differential ultracentrifugation-based techniques, size-based techniques, immunoaffinity capture-based techniques and exosome precipitation[30,38]. Each method has advantages and disadvantages depending on the cost, reliability, and speed of isolation.[29,31]

Ultracentrifugation-based isolation techniques

The differential ultracentrifugation usually consists of a series of centrifugation cycles (~100,000 to 120,000 × g), that lead to a separation of exosomes from other components in a sample, based on the density and size differences of the particles. Prior to isolation, a cleaning step is necessary to be carried out for human plasma/serum in order to get rid of large bioparticles from the sample. Protease inhibitors are used to prevent the degradation of exosomal proteins.[47] Between runs, the supernatant is aspired and, depending on the centrifugal force applied, either the supernatant or the pellet is resuspended in an appropriate medium such as phosphate buffered saline (PBS). Subsequently, an increasing centrifugal force is used.[71,72] After that, the isolated exosomes are resuspended and stored at minus 80°C until further analysis. This method of isolating exosomes is also known as the pelleting method or simple ultracentrifugation method[18,23] There are two types of ultracentrifugation-differential ultracentrifugation and density gradient ultracentrifugation. In the isolation of exosomes, the use of density gradient ultracentrifugation has become popular. In this methode, the separation of exosomes is realized in a density gradient medium, based on their size, mass, and density. This process is accomplished in a centrifuge tube with progressively decreased density from bottom to top. A sample is layered as a narrow band onto the top of the density gradient medium. Upon applying a centrifugal force, solutes (including exosomes) move as individual zones through the density

gradient medium towards the bottom, each at its specific sedimentation rate. The separated exosomes can then be conveniently recovered by simple fraction collection. While a continuous gradient is used for analytical applications, a discontinuous gradient (stepped gradient) is more suited for preparative purposes in which the separated exosomes are located at the interface of the density gradient layers, facilitating their harvesting. Due to the heterogeneity of exosomes and size of extracellular vesicles, differential ultracentrifugation often suffers from contamination and exosome losses[46,47].

Size-based isolation techniques

Ultrafiltration

One of the most popular size-based exosome isolation techniques is ultrafiltration. Using membrane filters with defined molecular weight or size exclusion limits, exosomes can be isolated. Ultrafiltration is faster than ultracentrifugation and does not require special equipment. The use of force may lead to the deformation and breaking up of exosomes. To confirm the success of exosome isolation, Western blot can be used in order to detect exosomal biomarkers[58,65].

Exclusion chromatography

Another size-based separation technique applied in exosome isolation is size exclusion chromatography (SEC). In SEC, a porous stationary phase is used to select macromolecules according to their size. In a sample, components with small hydrodynamic radii are able to pass through the pores, as opposed to exosomes that have large hydrodynamic radii and are excluded from entering the pores.[30,38,39] Examined by transmission electron microscopy, the isolated exosomes are structurally intact[67]. Dynamic light scatttering and nanoparticle tracking analysis were used to determine the size distribution. Hence, exosomes were individualised by laser-highlighting while in Brownian-motion, and their size was inferred based on their velocity. Western blot can also be used to evaluate the quantity and purity of the isolated exosomes and to confirm the biomarkers associated with the isolated exosomes.[43]

Immunoaffinity capture-based techniques

Immunoaffinity capture is an excellent technique for isolating exosomes of a specific origin or even subpopulations of exosomes. This methode has a lower efficiency, because only a subset of exosomes expressing the antibody-recognized protein is captured, but with much higher purity than those isolated based on the physical properties of exosomes[16,21] Because the exosomes have an important content of proteins and receptors in the membrane, data showed new techniques for the isolation of exosomes by tapping on immunoaffinitive interactions between those proteins (antigens) and their antibodies. Exosome biomarkers for immunoisolation are membrane-bound, lacking soluble counterparts. ELISA, a microplate-based enzyme-linked immunosorbent assay, expresses the results as absorbance values and compares the expression of known surface biomarkers. The absorbance values can also be extrapolated to quantify the captured exosomes through calibration using standards with known exosome counts. This microplate-based immunoaffinity capture approach produced results comparable to those obtained by ultracentrifugation, using less sample volumes. All these observations reveal the superiority of immunoaffinity capture over ultracentrifugation. Immunoaffinity capture was developed with mass spectrometry. Antibodies were immobilized onto highly porous monolithic silica micropipette tips, in order to capture exosomes. CD9 was chosen as antigen because it is abundantly expressed on the surface of exosomes derived from different origins. An automated multichannel pipette system allowed the isolation of exosomes from 12 serum samples simultaneously within 10 min.[47,68,74]

Exosome precipitation

Using water-excluding polymers such as polyethylene glycol (PEG), exosomes can be settled out of biological fluids by altering their solubility or dispersibility. PEG ties up water molecules and forces less soluble components out of solution. Samples are incubated with a precipitation solution containing PEG with a molecular weight of 8000 Da. After an incubation at 4°C overnight, the precipitate containing exosomes is isolated either by low speed centrifugation or filtration. Exosome precipitation is easy to use and does not require any specialized equipment. More commercial kits for the isolation of the exosomes compatible with body fluids including serum, plasma, ascites, urine, cerebrospinal fluid, and culture medium have been released (Exo□Quick TM, System Biosciences). The isolation process involves a simple one-step precipitation. This methode has advantages with

respect to the processing time, but the result may not be equivalent to that obtained by ultracentrifugation. The purity of exosomal proteins can be evaluated by Western blot and RNA quantification by qRT-PCR. Some total exosome isolation kits developed offer reagents for cell culture medium, urine[9,37], serum, and other body fluids like cerebrospinal fluid, ascitic fluid, amniotic fluid, milk, and saliva, as well as a kit for plasma. Total RNA and protein can then be purified using a total exosomal RNA and protein isolation kit.[60,71,72]

EXOSOMES' FUNCTIONS

Exosomes contain a wide variety of molecules, including proteins, RNAs, lipids, and fragments of genomic DNA that are present in the parent cell[13,18] Their protein carrier profile (surface proteins, intracellular proteins) can provide information about the physiological state of the cells they originate from.[69] Studies report that these proteins can serve as biomarkers for the isolation and quantification of exosomes, thus distinguishing them from other vesicles.[10,43,44] Outside of the cell, exosomes can act locally or by entering the blood flow, at distance from the site of origine. Somes may interact with the host cells either by complete internalization into the cell, or by direct fusion with the host cell membrane. Denzer *et al.* (2000), implied that exosomes do not necessarily require internalization to exert their action and illustrated that follicular dendritic cells are able to activate immune cells using MHC–peptide complexes and other proteins that they do not express, but which can be found on the exosomes carried on their surface. The cell-cell communication is based on diverse mechanisms of phagocytosis and endocytosis.[36,40,42] Those mechanisms depend on exosome dimension and exosome cargo. Signal transfer requires vesicle fusion with the host cell membrane.[11] This can occur either via direct fusion with the cell membrane or a 'back-fusion' step in which the exosome is taken up by an endocytic organelle. The later process is not entirely documented but some authors (Bissig *et al.* 2014) emphasize the role of lipid LBPA and protein Alix. Abrami *et al.*(2004) incriminated back-fusion as the escaping route of anthrax toxin lethal factor from endosomes to the cytosol. Various functions are taken into consideration for exosomes but the best-established ones are their role in the immune responses. Up to date, Lo Cicero *et al.* (2015), described that the increased expression and activity of proteins within the melanosomes (keratinocyte-derived exosomes) modulate melanin synthesis, thus influencing skin pigmentation. Experiments in vitro showed that exosomes transfer not only proteins and lipids, but also mRNA and microRNA into acceptor cells. Results sugest that these RNAs have functional effects in recipient cells. This mode of action of exosomes has been a focus of special interest in cancer biology. Data showed that exosomes from breast cancer cell lines are enriched with miRNAs as opposed to nontumorigenic breast cell lines. Exposure of normal cells to exosomes derived from breast cancer cell lines improved cell survival and proliferation and decreased the expression of some tumour-suppressor proteins. Although exosomes contained a wide variety of molecules, miRNAs have been the center of attention due to their role in regulating gene expression. The exosomal miRNAs are transported from their parent cells and the exosomal profile is influenced by physiological conditions form their parent cells. The chromosome 19 miRNA cluster (C19MC) is a unique group of 58 miRNAs exclusively expressed in the human placenta and in undifferentiated cells.[19,70] Luo *et al.* demonstrated that release of C19MC miRNAs is *via* exosomes and one of the C19MC-encoded miRNA is involved in tumor necrosis factor (TNF)-α signal transduction. Data sugest that miRNA profile of whole blood and blood-derived exosomes obtained from patients with metabolic syndrome had similar expression of miR-17, miR-197, miR-509-5p, miR-92a, and miR-320a. It has been reported that the proportion of exosomal miRNAs is higher than that in their parent cells and the exosomal miRNA profile can differ from those of the parent cells. Exosomes are also involved in various functions of adipose tissue.[14,24] Adipose tissue-derived exosomes have been isolated from culture medium of adipose tissue, adipocytes, and adipose tissue-derived stem cells (ADSC). Data showed that both 3T3-L1 adipocytes and primary adipocytes secrete large proportions of exosomes. Katsuda *et al.* reported ADSC-derived exosomes that were larger, indicating that the size range of the exosomes may differ based on the cellular source of isolation.[72,74] Adipose tissue-derived exosomes can be characterized based on the presence of adipose tissue-specific markers, such as fatty acid binding protein 4 (FABP4; adipocyte differentiation marker) and adiponectin. Data ilustrated that the characterization of exosomes released pre- and

post-adipogenesis showed differences in the protein content. Pref-1 and FABP4 were decreased while adiponectin was increased in the post-adipogenesis exosomes, but there were no changes in the exosomal markers, such as CD9, CD63, TSG101, and Alix.[24,26] These observations lead to the idea that proteins, which are commonly used for bio-marking exosomes, can be used to identify the adipose tissue-derived exosomes.[43,44] The release of exosomes has been reported to vary depending on body weight.[4,32] The quantification of exosomes isolated from subcutaneous and omental ADSC of lean and obese donors suggests that ADSC from obese individuals secrete higher concentrations of exosomes. Exosomes isolated from adipose tissue explants derived from lean versus obese individuals showed a higher number of exosomes released by lean adipose tissue. The secretion of a higher amount of exosomes by lean adipose tissue may be explained by the fact that there is a higher number of adipocytes in lean adipose tissue. The release of adipose tissue-derived exosomes is influenced by the extracellular medium. Adipose tissue hypoxia is one of the dysfunctional processes seen in adipose hypertrophy which can cause dysregulated secretion of adipocytokines. Experiments in which 3T3-L1 adipocytes cultured under hypoxic conditions released a higher amount of exosomes, emphasize the idea that the nature or condition of their parent cell determines exosome secretion. The contents of adipose tissue-derived exosomes are similar to their parent cell and include various adipocytokines, such as adiponectin, leptin, resistin, TNF-α. Adipose tissue-derived exosomes are also enriched with enzymes, such as acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, FA synthase, and lipids.[14,24,26] Data report that the levels of these enzymes were found to be upregulated in obesity. [4,32] Analysis of the circulating vesicles from adipose tissue, before and after a reduced calorie diet intervention, evidenced a decrease in perilipin-A in the vesicles. This observation suggests that an analysis of the composition of adipose tissue-derived exosomes can reflect the metabolic state of the adipose tissue. Exosomes isolated in hypoxic conditions showed upregulated expression of lipogenic enzymes. The changes in proteomic content of adipose tissue-derived exosomes reflect the condition of obesity and its related comorbidities.[69] A characterization and quantification of the contents of the exosomes can provide the health status of the adipose tissue and reflect their involvement in various biological functions. Data demonstrate that adipose tissue-derived exosomes may be an underlying mechanism for the regulation of various biological functions and progression of various diseases. The treatment of a Huntington's disease cell line with ADSC-derived exosomes reduced the mHtt aggregates and saved the cells from apoptosis. The data show that exosomes can be involved in nerve regeneration. The exosomes inhibited neuronal cell death and promoted re-myelination and re-genesis of neurons. They also increased the viability of the neuron-like cells expressing amyotrophic lateral sclerosis mutation. Adipose tissue-derived exosomes play a role in immune regulation. Exosomes from ADSC affected the proliferation rate of T cells and inhibited the activation by reducing the secretion of IFN-γ. There is also data that suggest that adipose tissue-derived exosomes can promote tumor growth. The treatment of the MCF7 breast cancer cell line with exosomes derived from ADSC showed greater migration *via* activation of the Wnt signaling pathway[14,24,26]. The melanoma cells incubated with exosomes secreted by the 3T3-F442A exhibiting cells enhanced migration capacity. Because adipose tissue-derived exosomes are involved in the progression and development of tumors they can be a target in cancer treatment. Cancer therapy is an area of interest and adipose tissue-derived exosomes have been used as carriers of specific cargo. Adipose tissue-derived exosomes are involved in metabolic regulation, data demonstrated in studies in which monocytes were incubated with adipocyte-derived exosomes. This resulted in differentiation of the monocytes into macrophages with upregulation of pro-inflammatory genes. The macrophages also inhibited phosphorylation of Akt in the adipose tissue[14,24,26]. The inflammatory role of adipose tissue-derived exosomes is exaggerated in obesity and plays an important role in development of obesity-related diseases and systemic insulin resistence (IR). The studies demonstrate that adipose tissue-derived exosomes and their content can mediate gene regulation and functioning in distant cells. In obese pregnancies, adipose tissue-derived exosomes may communicate with the placenta and induce changes in its function, which may contribute to the development of GDM (Gestational Diabetes Mellitus). Data suggests that adipose tissue-derived exosomes can be an important factor in the pathogenesis of GDM. Wang *et al.* ilustrate in their experiment, in which they use pancreatic cancer-derived exosomes, that exosomes which entered skeletal muscle cells,

initiated lipidosis and inhibited glucose uptake. Exosomes also downregulated the insulin and PI3K/Akt signaling pathway and affected the activity of glucose transporter (GLUT4). IR in muscle cells was observed after co-incubation with macrophages treated with adipose tissue-derived exosomes. Data suggests that adipose tissue-derived exosomes could mediate the onset of metabolic disease. Other studies reviewed suggest that exosomes secreted by cells from metabolic tissues can influence metabolism among tissues and be an initiator of diabetes and GDM during pregnancy.

EXOSOME SIGNALLING

In order to apply their biological functions, exosomes must release their contents into the new host cells. [27,28,31] Endocytosis of exosomes is *via* the exosomal trafficking pathway.[63] The endocytosis process can occur through different mechanisms such as phagocytosis, receptor and raft-mediated endocytosis. The phagocytosis mechanism occurs mainly in phagocytic cells. Feng *et al.* demonstrated that RAW 264.7 macrophages cells internalize exosomes derived from K562 and MT4 cell lines and the internalization was actin-mediated. It was also mediated by phosphatidylinositol 3-kinase (PI3K) and dynamin2. Tian *et al.* document that pancreatic cancer cells internalize exosomes that fusioned with endosomes of the recipient cell, potentially facilitating the transport to neighboring cells. The mechanism of receptor-mediated endocytosis may be *via* the classical or non-classical pathway. The former occurs *via* caveolin or clathrin membrane proteins. Data shows that exosomes derived from virus-infected cells are internalized by target cells *via* caveolin-dependent endocytosis. It is reported that knockdown of the CAV1 gene lead to significantly reduced exosome uptake so this observation demonstrated the caveolin-mediated endocytosis. Bone marrow-derived mesenchymal stromal cells take up PC12 cell-derived exosomes *via* clathrin-mediated endocytosis. Researchers on uptake of macrophage-derived exosomes by human trophoblast cells showed that uptake is an endocytic process mediated by clathrin and the uptake of exosomes induced secretion of pro-inflammatory cytokines by the placental cells. This observations suggest there is a change in placental phenotype induced by exosomes. The non-classical endocytic uptake of exosomes appears to be independent of membrane proteins. It has been reported that exosome uptake by glioblastoma cells is done *via* lipid raft-mediated endocytosis and is mediated by extracellular signal-regulated kinase-1/2 and HSP27. Exosomes can also interact with other cells by their adhesion to a potential docking site found on target cells. This way of interaction is mediated by the presence of transmembrane proteins on the surface of the exosomes. Dendritic cell-derived exosomes express intercellular adhesion molecule-1, major histocompatibility complex and co-stimulatory molecules which allow the exosomes to interact with target cells *via* their respective signaling receptors.

PATHOLOGICAL CONDITIONS CAN INFLUENCE EXOSOMES RELEASE

Data reported that pathophysiological conditions and stress can affect exosome biogenesis and release. Recent research shows that exosomes exposed to physiological change or pathological stimuli suffer modification of proteomic profile and RNA profile, thus offering a mirror of the microenvironment from parent cell.[53,61] A classical phenotype of several diseases (such as ischemic CVD, malignancies of diverse origins, obesity, preeclampsia, physiological challenges such as pregnancy) is hypoxia or low oxygen tension which is a stress-induced physiological condition. Hypoxia induces the activation of hypoxia-inducible factor (HIF), a key factor in the cellular adaptation to low oxygen concentrations.[50] HIF is a major modulator of exosome biogenesis and HIF-mediated intercellular exosome signaling is present in many physiological and pathological conditions.[35,70] The cellular response to hypoxia is to increase endothelial cell migration and angiogenesis. Data suggests that exosomes are involved in these vascular changes. Angiogenic ability of exosomes has been proved in studies on aggressive tumors. The biogenesis and release of exosomes is also affected by glucose concentration. Researchers on the effects of glucose on exosome release showed that there is an elevated number of exosomes from trophoblast cells cultured under both high and low glucose concentration. The exosomes released in these conditions induced secretion of pro-inflammatory cytokines from endothelial cells. The data may infere that this mechanism potentially mediates the maternal pro-inflammatory profile seen in pregnancies with glucose intolerance. The exact mechanism of alterations in exosome biogenesis

and of exosomal miRNA profile under different extracellular glucose concentration is not completely understood. There is evidence that changes in intracellular Ca^{2+} concentration may play a vital role in membrane trafficking, fusion, and retrieval and also play a role in modulating exosome release in response to extracellular glucose. Similar data has been reported by Saez et al (2018) and Salomon *et al.,* 2013 who stated that the release of placenta-derived exosomes in the foetoplacental vascularization in GDM (gestational diabetes mellitus) is mediated by oxygen partial pressure and glucose concentration and the exosomes alter their cargo, leading to endothelial dysfunction and activation. GDM is defined by the onset of glucose intolerance of variable severity, first recognized during pregnancy. Foetoplacental endothelial dysfunction is characterized by changes in the L-arginine–adenosine signalling pathway and inflammation. Since there are numerous clinical implications, the better understanding of these mechanisms may result in possible therapeutic strategies to promote vascular health in the children from mothers with GDM. Due to changes in pregnancy-related hormones that occur during early gestation, an increase in insulin resistence is frequent. A healthy pregnancy's outcome is highly reliant on the placenta, a very complex and multifunctional materno-fetal organ that acts as a nutrient sensor, controlling maternal–fetal nutrient transport and also as a nutrient sensor which detects maternal–fetal nutrient status. The placenta is also a transient endocrine organ which secrets various hormones and cytokines that can control maternal and fetal metabolism and is made up of trophoblast cells, specifically the cytotrophoblast, syncytiotrophoblast (ST), and extravillous trophoblast (EVT).[59,8,15] Data shows that placenta secretes large quantities of exosomes, which carry proteins, miRNAs, phospholipids that play crucial roles in maintaining feto–maternal communication, in mediating cell-to-cell communication and inducing different effects on target cells depending on the cell origin and exosome content. The researchers suggest that it may contribute to the pathogenesis of GDM. Recent studies highlight the utility of exosomes in the diagnosis of GDM, in identifying its onset and treatment monitoring.[36,41,47,86] Women with GDM have a distinct exosomal profile when compared to the profile of healthy pregnancies. Placental-derived exosomes can be isolated in the maternal circulation using the immunoreactive placental protein PLAP. PLAP is an integral membrane protein (enzyme) unique to the placenta produced mainly by syncytiotrophoblast. Using immunohistochemistry stain for PLAP, the majority of chorionic trophoblastic cells are positive for PLAP. During the first trimester of pregnancy, the release of placental exosomes into the maternal blood may result from extravillous trophoblast and/or syncytiotrophoblast cells.[17] Placental derived exosomes are secreted as early as 6 weeks of gestation in concentrations varying according to the stage of gestation[51,58] . The total number of exosomes measured in maternal plasma between 11 and 14 weeks of gestation is up to twofold greater in women who later developed GDM (diagnosis at 22–28 weeks) compared to those who had a normoglycaemic pregnancy. Data showed that an elevation in total exosome concentration in maternal plasma is correlated with maternal BMI, but the ratio of $PLAP^+$ to total exosome number decreases with higher maternal BMI across gestation. In GDM, the augmentation in exosome numbers is due to an increase in total exosomes other than $PLAP^+$ exosomes[64,66] Obesity is an underlying mechanism for the development of GDM and the IR seen in obesity is maintained by the adipose tissue, which releases adipocytokines and exosomes that mediate various metabolic disorders. In addition, adipose tissue-derived exosomes are altered in metabolic disorders. Data suggests that the dysregulated secretion of adipose tissue-derived exosomes has an role in the development of GDM in obese mothers.[24,26] Plasma exosomes isolated from obese and GDM subjects induce the secretion of pro-inflammatory cytokines from endothelial cells that explain the inflammatory phenomena typically associated with GDM. In addition to their physiological role, changes in exosome concentration and/or content may be of clinical utility in the diagnosis of placental dysfunction. Abnormalities in the levels or in the composition and molecular cargo of placenta-derived exosomes are associated with pregnancy complications such as preeclampsia, gestational diabetes mellitus, and pre-term birth (Murray *et al.*, 2015; Salomon *et al*., 2014). Decreased levels of placental exosomes have been found in the plasma of pregnant women who had pre-term birth when compared to the plasma levels of women who had normal deliveries (Murray *et al.*, 2015). Vargas *et al.* (2014) demonstrated that the protein syncytin-2 was decreased in the exosomes of women with

preeclampsia during their second and third trimester. Therefore, placental exosome levels, as well as their molecular composition in the plasma of pregnant women, may be important in predicting different complications during gestation.

EXOSOMES-DERIVED BIOMARKERS CAN BE POTENTIAL DIAGNOSTIC MARKERS OF DISEASES

Over the past decades, studies have demonstrated that exosomes contain diverse types of proteins including common membrane and cytosolic proteins as well as origin-specific subsets of proteins reflective of cell functions and conditions.[28,53] An increasing number of exosomal proteins have been found to be potential biomarkers for a variety of diseases including cancer, as well as liver and kidney diseases. Biomarkers are universally defined as biochemical alterations that can be objectively detected and quantified in human tissue, cells, or fluids and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention.[7,57] These criteria should be met whentaking into consideration potential biomarkers derived from exosomes in order to develop an efficient clinical test for early diagnosis of diseases. Exosomes can be isolated from easily attainable biofluids such as blood and urine, making them very attractive targets for diagnostic application.[71,72,37] CD63 is a member of the tetraspanin scaffolding membrane proteins family that exosomes are highly enriched in. Logozzi *et al.* reported in 2009 that plasma $CD63^+$ exosomes are significantly increased in melanoma patients compared to healthy controls. CD81, another exosomal marker from the tetraspanin family, plays a critical role in hepatitis C and is associated with inflammation and severity of fibrosis, suggesting that exosomal CD81 may be a potential marker for hepatitis C diagnosis and treatment response. A number of exosomal protein biomarkers have been found to be potentially useful in the diagnosis of central nervous system diseases such as glioblastoma, brain tumors, Alzheimer's disease (AD). Studies have also shown that α-synuclein, whose aggregation plays a central role in Parkinson's disease pathology, is released in exosomes. Proteins in urinary exosomes, which are easily attainable by noninvasive means, have also been exploited for potential utility in diagnostics, especially for urinary tract diseases[9,37]. In 2006, Zhou *et al.* found that urinary exosomal fetuin-A is increased in intensive care unit patients with acute kidney injury (AKI) compared with patients without AKI. Urinary exosomal proteins have also been investigated as potential biomarkers for bladder cancer and prostate cancer. Early detection and diagnosis of prostate cancer may be achieved using the prostate-specific antigen (PSA) test but this has low specificity and a high false-positive rate, which can lead to overtreatment of indolent prostate cancers. New markers with a higher diagnostic accuracy are much needed for prostate cancer so in 2008, Mitchell *et al.* reported that the level of circulating miR-141 is a robust diagnostic marker for prostate cancer. Brase *et al.* showed that serum levels of miR-141 and miR-375 are correlated with tumor progression in prostate cancer so exosomal miRNAs may be valuable markers for prostate cancer diagnosis. Exosomal miRNAs have been most frequently exploited as biomarkers for cancer diagnosis. In 2008, Taylor and Gercel-Taylor reported that eight miRNAs can be diagnostic markers for ovarian cancer. Exosomal miRNAs also show potential as biomarkers for the diagnosis of esophageal squamous cell cancer (ESCC). In 2013, Tanaka *et al.* reported that the exosomal miR-21 level is elevated in serum from patients with ESCC and is positively correlated with tumor progression and aggressiveness.

There are also studies that suggest that bodily fluids other than serum and urine may serve as alternate sources for diagnostic exosomes. Palanisamy *et al.* reported in 2010 that human saliva contains hundreds of stable mRNA core transcripts, which may be exploited as a possible resource for disease diagnostics. In 2013, Lau *et al.* showed that saliva exosomes may provide discriminatory biomarkers for pancreatic cancer . Amniotic fluid is another bodily fluid that has been investigated as a potential source of diagnostic exosomal markers and in 2007, Keller *et al.* suggested that exosomes from amniotic fluid may be potentially used in early prenatal diagnostics. Gilad *et al.* reported in 2008 that miRNAs associated with the human placenta (miR-526a, -527, -515-5p, and -R521) are detectable in both the serum and amniotic fluid of pregnant women and are correlated with the pregnancy stage.[59,64,66,73]

CONCLUSIONS

Exosomes are a prominent research interest due to to their role in intracellular communication and

signaling.[11,40,42,54] In the exosome research domain the most important issue is understanding the biological significance of these structures. There is no sufficient data about their basic physiological functions so it is difficult to understand how exosomes have been implicated in the pathogenesis of different diseases. It is important to mention that publications in the field of exosomes show a great interest in criteria used for distinguishing them from other extracellular vesicles in order to avoid confusing the domain which would encourage scepticism. The study of exosomes provided many data about their role in intercellular communication and many studies emphasize that they may act as vehicles for "bad" communication or spread.[54] As was the case of miRNAs in cancer, exosomes contain numerous disease-associated cargos (for example, neurodegenerative-associated peptides, (in Alzheimer's disease), tau (in neurodegenerative diseases), prions (in transmissible spongiform encephalopathies), alpha-synuclein (in synucleinopathies, including Parkinson's disease) and superoxide dismutase 1] (in amyotrophic lateral sclerosis).[19,70] The mechanisms by which disease-associated factors spread between cells is not completely understood and exosomes would provide a way for such transmission. The presence of exosomal proteins, such as Alix, in association with Alzheimer's senile plaques emphasize the idea that exosomes can be a mediator in such a spread. The hope is that having a way to control exosome release and spread may be useful in combatting some of these diseases. The studies showed that exosomes transport bioactive molecules, such as proteins, lipids, mRNAs, and miRNAs. Exosomal miRNA play an important role in the transfer of the genetic material from one cell to another. This functional mechanism has important relevance in the pathogenesis of various diseases, particularly obesity and GDM. [32,41,49] Another important role of exosomes is their involvement in physiological processes, including intercellular communication between endothelial cells and inflammatory cells. Data ilustrated that high plasmatic d-glucose increased the number of exosomes released and altered the exosome protein and RNA composition in endothelial cells and demonstrated a role for exosomes in GDM and also in the maternal and foetal circulation. Exosomes isolation techniques showed a great progress and the occurrence of exosomes in easily available body fluids such as urine and saliva makes this domain. promising in replacing conventional invasive prelevation techniques (such as using blood and cerebrospinal fluid) [29,71,72]

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