European Review for Medical and Pharmacological Sciences 2017; 21: 2940-2956

Exosomes as biomarkers and therapeutic tools for type 1 diabetes mellitus

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Abstract – Early diagnosis of diabetes mellitus can significantly improve therapeutic strategies and overall health span. Identifying biomarkers as a tool for determining the risk of developing diabetes as well as a monitoring strategy for progression of the disease state would be useful in predicting potential complications while simultaneously improving our ability to prevent and treat diabetes. Extracellular vesicles (EV) have recently emerged as prominent mediators of intercellular communication and as a potential source for the discovery of novel biomarkers. A deeper understanding of the cargo molecules present in EVs obtained from type 1 diabetes mellitus (T1D) patients may aid in the identification of novel diagnostic and prognostic biomarkers, and can potentially lead to the discovery of new therapeutic targets.

Key Words
Exosomes, Type 1 diabetes, Biomarkers, Therapy, Islets.

Introduction

Diabetes mellitus refers to a group of metabolic disorders characterized by elevated blood glucose levels attributed to an ineffective, insufficient or absent production of insulin. Long-term complications of the disease have been associated with macro and microvascular problems, leading to heart diseases, stroke, blindness and kidney diseases. Accurate monitoring of the progression towards these complications and responses to therapy at early stages, will allow for improvement in preventive and therapeutic strategies. Biomarkers can be objective indicators of the medical conditions of a patient, which can be measured accurately and reproducibly. The ideal biomarker for diabetes should detect disease trait (risk factor or risk marker), pathogenesis, disease state (preclinical or clinical), or disease rate of progression and prevent disease onset or help assess the possible therapies[1]. Extracellular vesicles (EVs) are small vesicles that have emerged as important mediators in cellular communication[2], which contain proteins, DNA and RNA species, (miRNA, mRNA, tRNAs, etc.)[3,4] as well as lipids and metabolites. Specific sources of EVs carrying distinct cargos have been associated with both normal physiologic and disease states. Their content can reflect biological events and disease progression[5]. This review focuses on the potential use of EVs as a novel class of biomarkers relevant to type 1 diabetes mellitus.

Classification of extracellular vesicles

EVs are a heterogeneous population of membrane bound structures released by cells into the extracellular space. EVs are released from most cells types [6] and can be isolated from most body fluids such as serum, plasma, urine, saliva and cerebrospinal fluid. Their potential role for diagnostic and therapeutic clinical use is currently under investigation. A consensus nomenclature for these heterogeneous vesicles is not fully established. However, EVs recently have been classified based on their tissue/cell-specific origin and size (Table I) or their biogenesis (Table II).

Microvesicles

Microvesicles arise through direct outward membrane protrusion, budding, and fission of the plasma membrane and subsequent release of the vesicles into the extracellular space [7]. Microvesicles can range from 50-1000 nm in size and are heterogeneous in shape. Several molecular localized changes undergo in the plasma membrane that result in the formation of the microvesicles, including changes in lipid, protein composition and

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Table I. Classification of extracellular vesicles based on tissue/cell-specific origin.

Type of EVs	Cellular origin	Size (nm)	Markers	References
Cardiosomes	Cardiomyocytes	40-300	CAV-3, flotillin-1	86
Ectosomes	Neutrophils or Monocytes	100-1000	CD14, TyA and C1a,	87, 88
Microparticles	Platelets in blood (PMPs) or endothelial cells (EMPs)	100-1000	Both CD31 PMPs: CD42	89
Oncosomes	Malignant cells	1000-10000	CAV-1	90
Prostatosomes or prostasomes	Seminal fluid	30-200	CD48, CD244	91
Tolerosomes	Serum antigen-fed mice, intestinal epithelial cells	40	MHC II, CD68 and LAMP1	92
Vexosomes	Adeno-associated virus vectors (AVV)	50-200	AAV capsids	93

calcium levels[8]. These are unique mechanisms of EV formation in comparison to exosomes, which are formed intracellularly within multivesicular bodies. This novel mechanism of microvesicle formation results in the regulated release of EVs containing specifically enriched molecular cargoes.

Retrovirus-like particles (RLPs)

Retrovirus-like particles (RLPs) are those EVs that resemble retroviral vesicles, but are non-infectious because they do not contain the full complement of genes required for cellular entry or viral propagation[9]. RLPs contain a subset of retroviral proteins, they are released from cells after a viral infection. Certain viruses can use RLPs to facilitate their propagation and entry into neighboring cells[10].

Apoptotic bodies or blebs

Apoptotic bodies or blebs are the largest EVs with a size range of 50-5000 nm. Apoptotic bodies are vesicles produced from cells undergoing programmed cell death or apoptosis. Apoptotic bodies bud directly from the plasma membrane like microvesicles and contain fragmented nuclei as well as fragmented cytoplasmic organelles[11].

Exosomes

Exosomes (EXOs) are small (about 30-200 nm in diameter) lipid vesicles derived from multivesicular bodies that are released by most cell types. EXOs are present in different body fluids including serum, urine, cerebral spinal fluid, and saliva and bronchiolar lavage fluid. EXOs have emerged as important mediators in cell communication, transferring proteins, lipids, DNA and RNA species (miRNA, mRNA, tRNAs, etc.) between cells[2,12]. Given that the cargo of EVs reflects the status of the cell type of origin there is a growing interest in the potential of EVs, in particular, exosomes, for diagnosis and therapy in multiples diseases[4,13].

Table II. Classification of extracellular vesicles based on biogenesis.

Type of EVs	Origin	Size (nm)	Markers	Content
Exosomes	Endolysosomal pathway	30-200	Tetraspanins: CD63, CD9, CD81. ESCRT components: TSG101, flotilin, Alix	mRNA, microRNA, non-coding RNAs, cytoplasmic and membrane proteins, including receptors and major histocompatibility molecules
Microvesicles	Cell surface	50-1000	ARF6, VCAMP3, integrins, selectins, CD40 ligand	mRNA, microRNA, non-coding RNAs, cytoplasmic and membrane proteins, including receptors
Retrovirus like particles	Plasma membrane	75-100	gag	Retroviral particles env, rec, and pol
Apoptotic bodies or blebs	Cell surface	50-5000	Thrombospondin, C3b, Annexin V, phosphatidylserine	Nuclear fractions, cell organelles

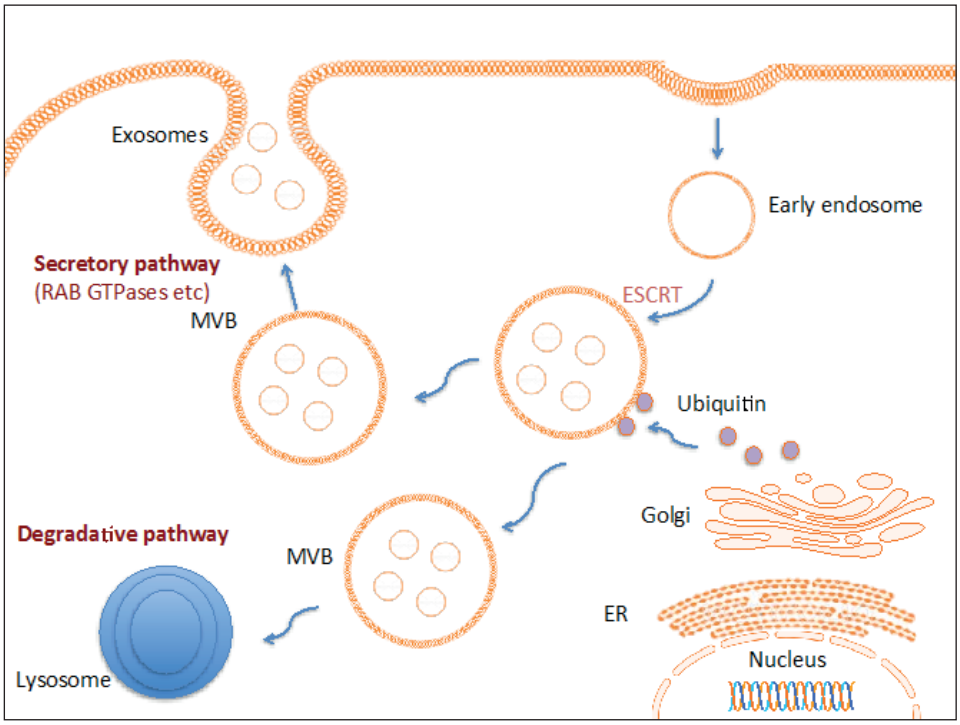

Figure 1. Biogenesis of exosomes.

Exosome biogenesis

Exosomes are secreted vesicles that originate within the multivesicular bodies (MVBs) as part of the endosomal network (Figure 1). Early endosomes fuse with endocytic vesicles and incorporate their content into those destined for recycling, degradation, or exocytosis[14]. The contents destined for recycling are sorted into recycling endosomes, or targeted for lysosomal degradation by ubiquitination and ubiquitin-dependent interactions with Endosomal Sorting Complex Required for Transport (ESCRT) machinery[15]. The ESCRT has four main complexes (ESCRT-0, -I, -II and -III), ESCRT is responsible for final delivery of ubiquitinated proteins to the degradation machinery. The remainder of the early endosomes then undergo a series of transformations to become late endosomes[16]. During this transformation, contents fated to be degraded or exported are preferentially sorted into 30-200 nm vesicles (ILVs) that bud into the lumen of late endosomes also known as MVBs[17]. In this process, the endosome membrane is reorganized and becomes highly enriched in tetraspanins such as CD9 and CD63, which play a critical role in exosome formation[18]. Also, the ESCRTs are recruited since they are required for the transport and the accumulation and sorting of molecules into ILVs[19]. Finally, the MVBs are targeted to either fuse with lysosomes or the plasma membrane which results in the secretion of the 30-200 nm vesicles into the extra-cellular space known as exosomes. Different mechanisms have been proposed for the release of exosomes. A number of Rab GTPases, including RAB11 and RAB35, or RAB27A and RAB27B, are recognized to play an important role in exosome release[20]. In addition, some exosomes may be released through budding from the plasma membrane independently of Rab GTPases.

Exosome composition

Exosome content is highly heterogeneous because exosomes can contain proteins, nucleic acids (DNA and RNA species such as miRNA, mRNA, rRNA, tRNA, scaRNA, snoRNA, snRNA and piRNA) and lipids (Figure 2). There are specific databases compiling published data containing exosome composition: EVpedia[21], Exocarta[22] and Vesiclepedia[23].

Lipid content

EXO membranes consist of a bilayer lipid membrane similar to that of cell plasma membrane. EXO membranes are not identical to the parent cell, but is depleted or enriched in specific

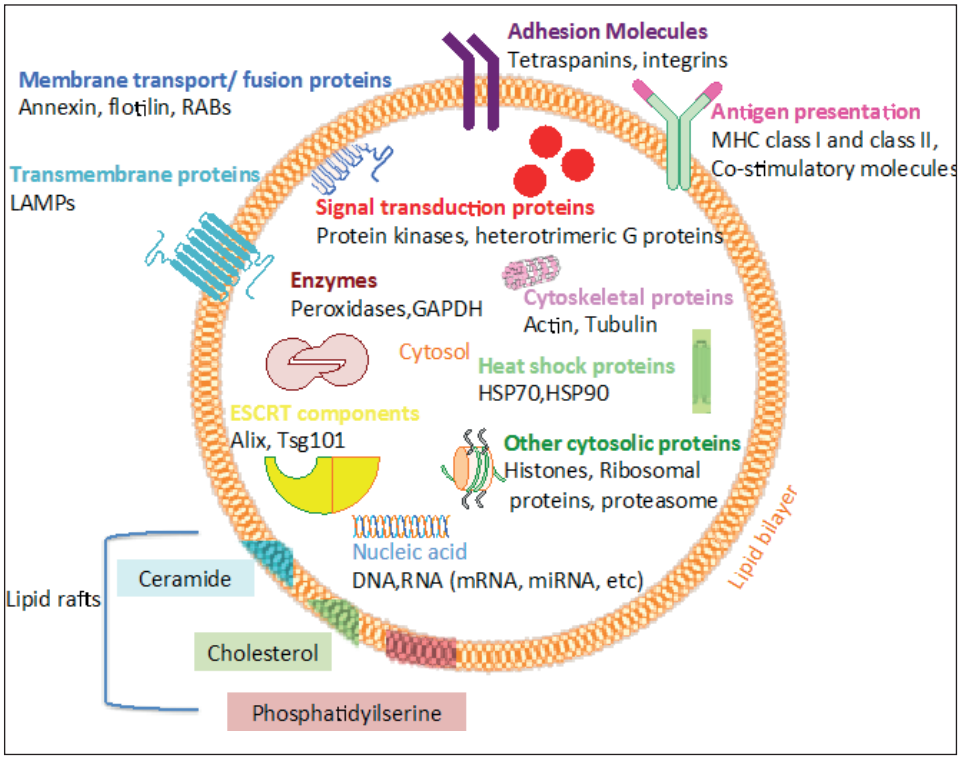

Figure 2. Schematic image of the molecular composition of an exosome.

lipids. EXOs contain sphingomyelin, gangliosides, cholesterol and desaturated lipids[24]. EXOs also contain phosphatidylserine in the outer leaflet, which facilitates their internalization by recipient cells[25]. In addition, EXOs contain enzymes involved in lipid metabolism and receptors and adhesion molecule and the lipid composition of EXOs derived from different cell sources can vary widely.

RNA content

EXOs contain diverse RNA species such as miRNA, mRNA, rRNA, tRNA, scaRNA, snoRNA, snRNA and piRNA[26], but are enriched in smaller, non-coding RNAs compared to the parental cell. Defined sequence motifs[27] and post-transcriptional modifications[28] in the 3' end in miRNAs may promote specific miRNA and mRNA packaging into EXOs. The EXO RNA content can be transferred to recipient cells where it plays a functional role[2,29].

DNA content

EXOs can transport DNA (exoDNA) that reflects the parental cell genomic DNA (gDNA)[30]. Recent studies have shown that both mitochondrial DNA (mtDNA) and fragmented chromosomal DNA are found in EXOs[31,32]. Furthermore, EXOs from plasma of cancer patients and cancer cell lines contain different double-stranded genomic DNA fragments containing the tumor-associated mutations[3,33]. Like RNA, this exosomal DNA also can be transferred between cells and can influence the function of the recipient cells, possibly playing important roles in pathological conditions[12,34].

Protein content

Proteins are found in the membrane or in the hydrophilic core of the EVs. These proteins include tetraspanins (CD9, CD63, and CD81 antigens), epithelial cell adhesion molecule (EpCam), and other adhesion proteins. The EXOs protein signature is unique and contained cell line-specific proteins, but in some instances, is related to the cell type and EXOs biogenesis[24]. For example, EXOs originated from the endosome compartment are more enriched in tetraspanins and major histocompatibility complex class II.

Exosome isolation methods

EXOs have been isolated from biological fluids including blood, urine, cerebrospinal fluid, tears, saliva and nasal secretions, ascites, and semen. All currently used protocols for purification co-purify different subtypes of EXOs (Table III).

Table III. Classification of extracellular vesicles based on biogenesis.

Method	Type	Principle	Advantages	Disadvantages	References
Ultracentrifugation	Differential	Sedimentation based on size and density	Gold standard	Time consuming Inability to separate exosomes from microvesicles Expensive equipment	35
	Sucrose-gradient	Flotation based on density	Removes protein contamination Collection morphological intact microparticles	Time consuming Inability to separate exosomes from microvesicles Expensive equipment	35
Affinity-based captured	Immunobeads	Separation based on affinity interactions	Fast Semi-quantitative characterization of the surface phenotype can be tissue-specific	Not suited for large sample volumes Captured extracellular vesicles may not retain biological functionality Co-purification of protein aggregates. Low yield.	35
Filtration	Microfiltration	Separation based on size	Easy and fast	Protein contamination Small sample volume limitations Inability to separate exosomes from microvesicles Exosomes can adhere to the filtration membranes and become lost. Also, since the additional force is applied to pass the analyzed liquid through the membranes, the exosomes can potentially be deformed or damaged.	35
Chromatography	Size-exclusion	Separation based on size	Collection morphological intact microparticles	Small sample volume limitations Time consuming Requires specialized equipment	36
Polymer precipitation	Polymer based	Separation based on Polyethylene glycol precipitation	Fast High yield Requires small sample amount	Low purity and specificity Protein contamination	38, 94
Microfluidic	Devices	Mechanics of fluid flow based	Increases throughput and allows multiplexing Reduced cost, sample size and processing time	Large sample volume limitations	95

There is no universal consensus as to the best method for isolation.

Ultracentrifugation

Ultracentrifugation is the most commonly used method to isolate EVs. This method consists of several sequential centrifugation steps, usually with the first steps being a 300 x g spin for 10 min followed by a 10.000 x g spin 30 min to eliminate intact cells, dead cells and cell debris. After depletion of cells and large apoptotic bodies by low-speed centrifugation, the EVs are pelleted in the final step at 100.000 x g for 70 min[35].

Sucrose-gradient centrifugation

Sucrose-gradient centrifugation is use in combination with ultracentrifugation to remove contaminating non-vesicular particles[35]. This procedure allows separation of vesicles according to their density, classically reported between 1.1 and 1.19 g/ml.

Affinity-based captured of extracellular vesicles

Affinity-based isolation enables the selective capture of specific subpopulations using antibodies to CD63, CD81, CD82, CD9, Alix, annexin, EpCAM and Rab5[35]. These could be used either alone or in combination. For this application, the antibodies can be immobilized on magnetic beads, chromatography matrices, plates, and microfluidic devices.

Size exclusion chromatography/ filtration

Separation of EVs based on the size is achieved through the use of chromatography[36] or microfiltration[37]. Column chromatography allows for sequential elution of EV size fractions from a single column. Microfiltration is used in combination with ultracentrifugation to eliminate dead cells, apoptotic bodies and large debris.

Polymer precipitation

Volume-excluding polymers such as polyethylene glycol (PEG) are used for the precipitation of extracellular vesicles, although the purity is lower than with some other isolation methods[38]. Contamination of EVs pellets with non-exosomal materials remains a problem for polymer-precipitation methods. In addition, the polymer substance present in the isolate may interfere with down-stream analysis.

Microfluidics

The application of these methods to biological fluids has not yet been described extensively[39]. However, recent advances in microfluidic-based technologies have made it possible to extract EVs from the blood in an easily reproducible, convenient manner. It is likely that this approach will become more prevalent.

Exosomes characterization methods

EXO quantification can be carried out directly or indirectly. There are a variety of methods (Table IV) that are currently used for analysis, but there is a lack of consensus in the field.

Protein quantification

The total protein content of the purified EVs can be determined by the Bradford assay, which also provides indirect quantification of EV[35]. However, the presence of proteins which are not associated to the EVs content can impact the total protein content and therefore bias EV concentration determination.

Table IV. Summary of exosome characterization methods.

Method	Characteristics	Advantages	Disadvantages	References
Protein quantification	Analysis of protein concentration (non-specific)	Easy and low cost	No specific information	35
Transmission electron microscopy	Assessment of morphology, size and markers	Direct evidence for the presence of EV	No quantification Need an expert in TEM	35
Scanning electron microscopy	Assessment of morphology, size and markers	For the presence of EV	No quantification Need an expert in SEM	96
Atomic force microscopy	Assessment of morphology and size	Direct evidence and quantification	Need an expert	41
ELISA	Specific EV proteins	Specific for exosome proteins	Unreliable Technical troubles	42
Nanoparticle tracking analysis	Analysis of absolute concentration of particles and particle size	Quantification	No distinguishment of EV from aggregated proteins	43, 96
Flow cytometry	Detection of EV markers	Detection specific EV markers	Low detection threshold	44, 97
Western blot	Detection of specific EV proteins	Detection specific EV subset	Cannot determine the presence of EVs	35

Transmission electron microscopy

Morphological examination is typically carried out using transmission electron microscopy (TEM) is an established technique that provides the direct evidence for the presence of EVs. EV suspensions are applied to grids, fixed, stained with osmium tetroxide or uranyl acetate, and contrasted by embedding in methylcellulose. EVs preparations examined by TEM show EVs with a cup shaped appearance, which is an artifact of the preparation procedure[40]. TEM can be combined with immunogold staining using gold conjugated antibodies to detect the presence of specific markers.

Scanning electron microscopy

Although TEM is considered a standard tool for characterizing the morphology of exosomes, scanning electron microscopy (SEM) is a newer, alternative approach to analyze EVs morphology and structure.

Atomic force microscopy

Atomic Force Microscopy (AFM) is used for topographic imaging of EVs. AFM also can be used to analyze the mechanical properties of nanoparticles[41].

ELISA

Alternatively, exosome ELISA kits (System Biosciences) allow investigators to quantify the number of exosomes based on the level of the exosome-associated proteins including CD9, CD63, and CD 81[42].

Nanoparticle tracking analysis and resistive pulse sensing

Nanosight™ nanoparticle tracking analysis uses light diffraction patterns to measure the size and the concentration of exosomes[43]. Similarly, direct quantification of exosomes can be performed using the qNano Gold (Izon Science) which measures nanoparticles using the tunable resistive pulse sensing (TRPS) principle, reporting concentration as a function of a defined size range. Both are label-free methods.

Flow cytometry

Traditional flow cytometry has been widely used for membrane marker identification[44]. Here it is recommended to assess not only the presence of selected membrane surface markers, but also the absence of contaminants, and to include appropriate isotype controls. There are inherent problems with this approach, as these instruments have traditionally been developed to measure whole cells, which are orders of magnitude larger than exosomes and contain more of each membrane protein. The use of antibodies coupled to beads can allow for detecting EXO surface proteins although not necessarily in a quantitative manner. Imaging flow cytometry has emerged as new alternative with increased fluorescence sensitivity, low background, and image confirmation ability[45].

Western blotting

Western blotting (WB) is a convenient method to detect the presence of surface markers include tetraspanins (CD9, CD63, CD81, and CD82), MHC molecules, and cytosolic proteins or cytoskeletal proteins. Isolated EVs are lysed, and the proteins are separated and analyzed [35]. However, WB alone cannot determine the presence of EVs.

Function of exosomes

Physiological function of exosomes

In the 1960s and 70s, studies suggested that membrane fragments and vesicles in the extracellular compartment or blood may have originated from specific or regular cellular activity[46]. In 1983, it was reported that vesicles harboring transferrin receptors were jettisoned by reticulocytes as part of their differentiation into red blood cells[47,48]. It was not until 1996, that exosomes were first shown to be involved in cell-to-cell communication and possibly play a role in antigen presentation[6]. More recent studies have focused on the potential functions of exosomes in different cell types. The function of exosomes depends on the origin and molecules packaged within. It has been observed that multiple cell types, including pancreatic islets of Langerhans, release EXOs that can transfer their cargos to target cells[49]. EXOs can transfer not only proteins, but also have been shown to transfer mRNA and miRNAs molecules which are taken up functionally by target cells[2]. This horizontal transfer of information via exosomes as a new mechanism of cell-to-cell communication has been reported in multiple cell models.

Exosomes in pathological processes: role in type 1 diabetes (T1D)

In addition to their biological function, exosomes are hypothesized to be involved in pathological processes and disease pathogenesis including cancer[5,34], virus[50,51] infection and autoimmune diseases such as type 1 diabetes mellitus[52].

Type 1 diabetes mellitus (T1DM)

Type 1 diabetes mellitus, also known as insulin dependent diabetes mellitus, is primarily a childhood associated autoimmune disease characterized by the destruction of insulin-producing ß cells in the pancreatic islets of Langerhans. Because of the autoimmune destruction of the insulin-producing ß-cells, there is an insulin deficiency and the body is unable to control blood sugar. Since insulin is the primary anabolic hormone that regulates blood glucose level, patients with T1D require the administration of exogenous insulin through multiple daily injections, guided by daily blood glucose measurements, for survival. Additionally, long-term type 1 diabetes due to the chronic hyperglycemia can lead to multiple complications including retinopathy, nephropathy, neuropathy and cardiomyopathy.

Pancreas and pancreatic islets of Langerhans

The pancreas is the organ in the body which functions as both endocrine and exocrine gland. Almost all the pancreas (95%) consist of exocrine tissue which is composed of acinar cells that produce digestive enzymes for digestion. The remaining is endocrine tissue comprised of large clusters of cells called islets of Langerhans, each containing thousands of cells. Islets are composed of different types of secretory cells: α-cells that secrete glucagon when glucose is low, β-cells that secrete insulin when glucose is elevated, δ-cells that secrete somatostatin to regulate α and β cells, PP cells that secrete pancreatic polypeptide, ε-cells that secrete ghrelin[53], neurons that produce AcCholine and Norepin, serotonin-producing enterochromaffin cells and gastrin-producing G1 cells[54]. Non-secretory cell types also are found within islets such as endothelial cells and immune cells. Due to the importance of the pancreas in regulating and balancing hormone levels in the body, damage or disease to the organ leads to severe metabolic imbalances, as in the case of diabetes.

Paracrine interaction within islets of Langerhans: exosomes

Paracrine interactions within the islet of Langerhans serves to orchestrate hormonal secretion and promote islet health and survival[55]. These paracrine interactions between cells within islets are mediated by peptides and neurotransmitters[56,57] secreted by pancreatic islet cells and, as very recently reported, by EXOs[49] (Table V). Thus EXOs are the emerging players that mediate paracrine communication between different cell types in islets[2,58]. Insulin-producing ß-cells lines[59,60] and islets[49] have been shown to release exosomes in the typical size range (30-200 nm size) and morphology. Interestingly, analysis of the content of islet-derived EXOs revealed the presence of insulin transcripts and insulin, C-peptide proteins, GAD65, low levels of glucagon and endothelial nitric oxide synthase, suggesting that these EXOs were mostly of insulin-producing ß-cell origin[49]. Furthermore, many miRNAs have been found to be enriched in EXOs derived from Islets of Langerhans[49] and insulin-producing ß-cells[59]. Islet-derived EXOs have the capacity to interact and transfer their content to surrounding cells such as endothelial cells[49]. Pro-inflammatory cytokines secreted by immune cells contribute to the immune attack of islets. Exposure of insulin-producing ß-cells and islets to pro-inflammatory cytokines, revealed that EXO release was induced containing protein involved in the TNF signaling pathway, autoantigens and immunostimulatory chaperones[61,62]. Moreover, insulin-producing ß-cells exposed to pro-inflammatory cytokines revealed an altered miRNA signature compare to non-cytokine-stimulated cells[59,61]. In addition, EXOs released by insulin-producing ß-cells exposed to pro-inflammatory cytokines have been shown to confirm apoptotic effects on recipient cells[59,63].

Exosomes and pathogenesis of type 1 diabetes mellitus

Type 1 diabetes mellitus is a tissue specific autoimmune disease, characterized by T-cell mediated destruction of the insulin-producing ß-cells. Although the initial triggering events of T1D are unknown, multiple factors likely are involved in the induction and pathogenesis of disease including genetic predisposing factors, exogenous infectious pathogens, non-infectious environment agents, endogenous antigens and physiological stress events[64]. HLA genes are the major risk genes for T1D, HLA class II gene variants play a major role controlling disease susceptibility via the presentation of autoantigen peptides to autoreactive T cells[65]. Antigen presentation is mediated by antigen presenting cells (APC). Recently, has been reported that Inflammation in the T1D pancreas play a role in modifying the presentation of autoantigen peptides from insulin-producing ß-cells[66]. Emerging evidence indicates that EXOs play a role in the

Table V. Summary of findings on exosomes released by insulin-producing ß-cells.

Year	Cell type	Species	Culture conditions	Primary isolation method	Findings	References
2016	Pancreatic Islets	Human and rat	Cytokines	Ultracentrifugation	ß-Cells Release Autoantigens GAD65, IA-2, and Proinsulin in Exosomes Together With Cytokine-Induced Enhancers of Immunity	62
2015	MIN6B1	Mouse	Cytokines	Ultracentrifugation	Transfer of exosomal microRNAs transduce apoptotic signals between ß-cells	59
2014	Pancreatic Islets	Human	Control	Ultracentrifugation	ß cell-endothelium cross-talk of extracellular vesicles released from human pancreatic islets	49
2014	Islet Stem Cells	Mouse	Control	Ultracentrifugation and Exoquick-TC kit	Exosomes released by islet-derived mesenchymal stem cells trigger autoimmune responses	52
2014	NIT-1	Mouse	Cytokines	Ultracentrifugation	EXOs isolated from the culture medium of INS-1 cells treated with cytokines at a low concentration inhibit apoptosis induced by a high concentration of cytokines	63
2014	MIN6	Mouse	Cytokines	Ultracentrifugation	ß-cell exosomes loaded with miR-29 stimulate TNF-alpha, IL-6, and IL-10 cytokine secretion from splenocytes isolated from diabetes-prone NOD mice in vitro	98
2012	NHI 6F Tu28	Rat	Cytokines Control	Ultracentrifugation	Specific proteins, specific sites of protein phosphorylation and N-linked sialylation in proteins associated with microvesicles from β-cells.	61
2011	MIN6	Mouse	Control	Ultracentrifugation	Insulinoma-released EVs are immunostimulatory and can activate autoreactive T cells spontaneously	67
2009	NIT-1	Mouse	Control	Ultracentrifugation	Insulinoma cells share some common properties with exosomes from lymphocyte and cancer cells and also differ from them in some properties	60

initiation of autoimmune responses in the islets. For example, rat and human pancreatic islets release intracellular ß-cell autoantigens (GAD65, IA-2, and proinsulin) in EXOs, which are then taken up by activated dendritic cells, a major antigen presenting cell (APC) type, to activate autoreactive T and B cells (Figure 3)[62,67-69]. Providing evidence that insulin-producing ß-cells released EXOs can cause and accelerate diabetes onset *in vivo* by stimulate the autoimmune responses[67,68].

Exosomes as Biomarkers in type 1 diabetes mellitus

Exosomes have emerged as a potential source of biomarkers for disease diagnosis and monitoring of therapeutic responses. The underlying autoimmune process of T1D occurs before the onset of clinical diabetes and this asymptomatic period provides an excellent opportunity for the prediction and prevention of the disease (Figure 4)[1]. However, there is a lack of suitable biomarkers for the identification and stratification of the high-risk population for specific intervention and lack of surrogate biomarkers to evaluate the efficacy of intervention in T1D. Currently the most useful biomarkers for T1D risk prediction are susceptibility genes and islet autoantibodies. Autoantibodies also are used as a predictive marker to identify subjects at risk, for diagnosis and during follow-up of patients. However, autoantibodies appear relatively late in the disease process, thus limiting their value in early disease prediction. The major autoantibodies in T1D are GAD65, IA-2 also known as ICA512, and in-

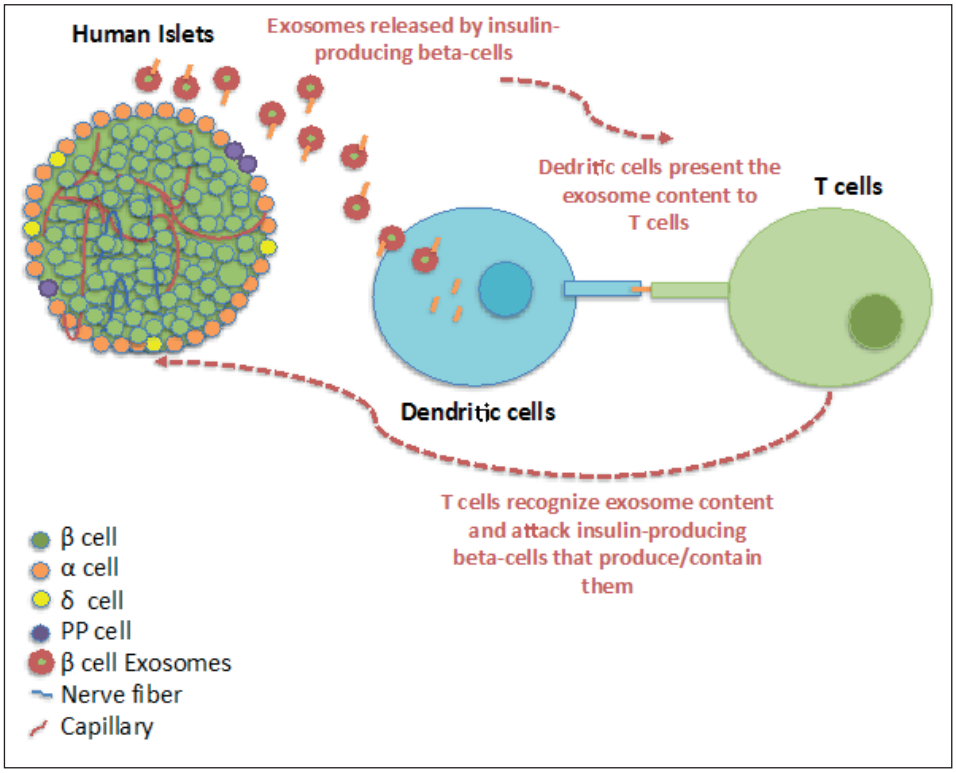

Figure 3. Summary Role of possible role of exosomes in type 1 diabetes pathogenesis.

sulin. GAD65 autoantibody positivity tends to be stable, IA-2 autoantibodies tend to decrease with disease duration and insulin autoantibodies cannot be used after initiation of insulin therapy. The risk associated with subjects carrying autoantibodies to two or three of these autoantigens. Genetic factors have been implicated as triggers in the pathogenesis of T1D, especially HLA genes that are involved in antigen presentation. Interestingly, the combination of high-risk HLA genes with autoantibodies further increases positive prediction. However, these markers do not fully meet the needs. Exosomes are released in physiological and pathological conditions. The EXO cargo is indicative of the state of the cells that can be potentially used for diagnosis. In numerous disease has been shown that healthy subjects and patients release exosomes with different content into the circulation, which can be measured as biomarkers. For instance, insulin-producing ß-cells release EXOs containing-specific miRNAs or proteins including autoantibodies that can be potentially used for T1D diagnosis (Table VI)[49,59,61,62]. Over the past few years, numerous studies have demonstrated that EXOs cargo is implicated in numerous diseases included metabolic diseases. In T1D context, EXOs have been used as biomarkers of numerous complications such as nephropathy[70-73] or retinopathy[71,72,74]. Moreover, recent studies have been shown that EXOs could be used for monitoring noninvasively islet transplantation outcome (Figure 4)[75]. Furthermore, EXOs provide advantages versus classical methods for the identification of biomarkers including: 1) EXOs can be easily isolated from biological fluids such as blood or urine; 2) EXOs are relatively stable and can be long-term stored at -80°C; 3) provide protease/nuclease controlled environment increasing molecule stability at the time; 4) allow for concentration of specific molecules of interest in easy to isolate particles; and 5) a subset can be isolated using a specific anti-cell surface marker antibody followed by analysis of specific cargo proteins/RNAs[4]. These characteristics make them very attractive for diagnostic applications in a clinical setting.

Exosomes as therapeutic tools in type 1 diabetes mellitus

EXOs also are potential novel targets for therapeutic intervention. EXOs are natural carriers of functional DNA, RNA and proteins[2,76] and can be used as a therapeutic delivery of these molecules and/or synthetic drugs[77]. However, systemic deliver of exosomes accumulates in the liver and spleen[78]. Here approaches to target EXOs to spe-

Table VI. Studies and exosomes biomarkers in type 1 diabetes mellitus.

Year	Specimen type	Species	Bio-markers	Primary isolation and validation method	Findings	References
2017	Plasma	Mice, Human	microRNAs	Chromatography and ultracentrifugation	Trasnplanted islet-derived exosomes biomarkers for monitoring rejection	75
2017	Urine	Human	AQP5, AQP2	Ultracentrifugation	Exosomes aquaporins as biomarker in type 1 diabetic nephropathy	99
2017	Urine	Human	Higher levels podocyte exosomes	Filtration	Biomarker of glomerular injury in T1D	70
2016	Blood	Rat	eNOS and caveolin-1	Centrifugation	Biomarkers for vascular injury	100
2015	Plasma	Human	Cytokines and angiogenic factors	Centrifugation	Biomarker for diabetic ocular complications	101
2015	Blood	Mouse	Higher levels endothelial exosomes	Flow cytometry	Biomarkers for arterial injury	102
2015	Urine	Human	Increase of cystatin B and alterations in protease profiles	Filtration	Biomarker of kidney injury in T1D	73
2015	Culture	Human	miR-126	Ultracentrifugation	Biomarker for diabetic retinophaty	103
2015	Urine and plasma	Human	Increased expression proteases	Ultracentrifugation	Biomarker for diabetic retinophaty	104
2015	Blood	Human	Increased number of platelet exosomes	Flow cytometry	Biomarker for microvascular complications in T1D	106
2014	Urine	Rat	Reduced expression of regucalcin	Immunoaffinity	Biomarker for diabetic retinophaty	106
2013	Urine	Human	miR-145	Ultracentrifugation	Biomarker for diabetic retinophaty	71
2013	Urine	Human	WT1 protein	Ultracentrifugation	Biomarker for diabetic retinophaty lymphocyte and cancer cells and als differ from them in some properties	72

cific cell types would enhance their therapeutic potential. Obtaining EXOs from genetically modified cells designed to load molecular contents and/or directional targeting molecules would allowing targeting of disease sites *in vivo*. EXOs have shown potential as a therapeutic agent in treating T1D (Table VII). In particular, EXOs from human urine derived stem cells can prevent kidney injury in T1D rats by the transfer of growth factors, transforming growth factor-ß1, angiogenin and bone morphogenetic protein-7[79]. Recent studies on EXOs from mesenchymal stem cells (MSCs), have been shown to have an immunomodulatory effect in culture through a T1D pro-inflammatory response[80,81]. Furthermore, delivery of specific miRNAs-using EXOs also can be a therapeutic tool. For example, EXOs from bone marrow stromal cell from diabetic rats conferred neuro-restorative effects in a rat stroke model by the transfer of miR-145[82]. In other studies, MSC derived exosomes have been used as small RNA carriers in T1D[83]. In addition to nucleic acids, exosomes have been successfully used to deliver drugs such as curcumin that had an effect on T1D mice after stroke ameliorating neurovascular dysfunction[84]. Current therapeutic alternatives to daily insulin injections

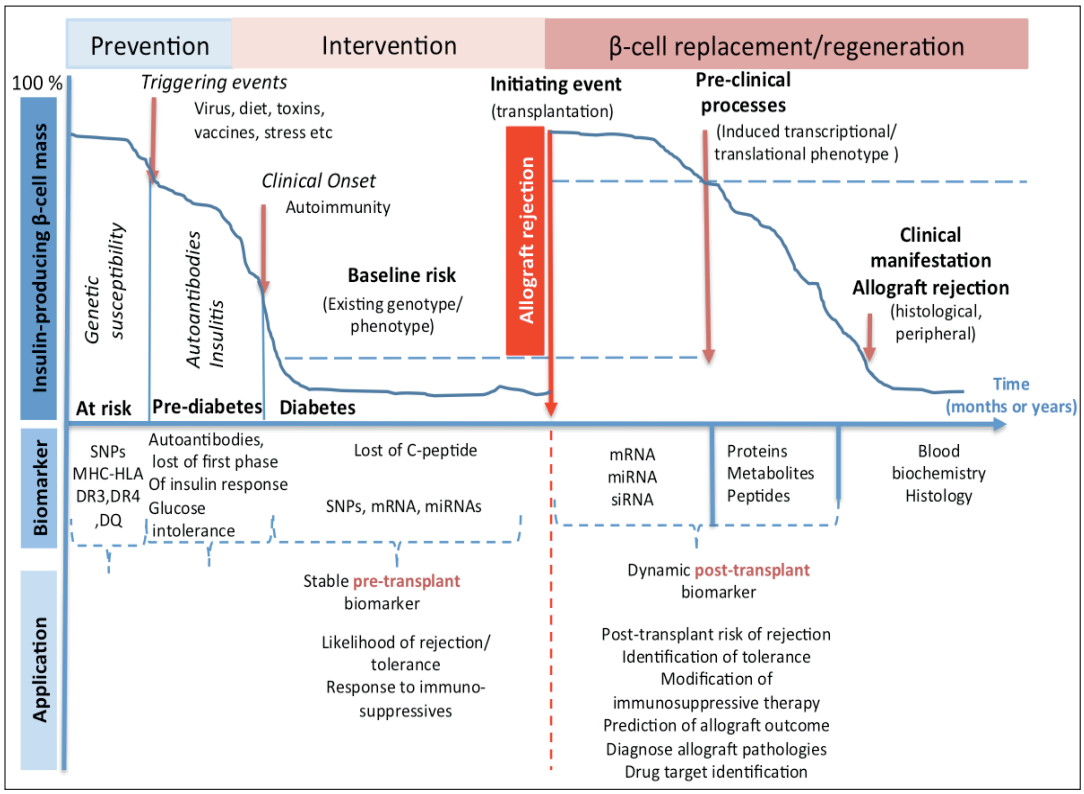

Figure 4. Biomarkers and opportunities for T1D prevention and intervention.

Table VII. Studies showing therapeutic applications of exosomes in type 1 diabetes mellitus.

Year	Mechanism	Findings	References
2017	Bone marrow exosomes transfer miR-106b and miR-222	Improve hyperglycemia	107
2016	Urine-derived stem cells transfer VEGF, TGF-β1, angiogenin and BMP-7	Polyuria improved in vivo and prevent apoptosis in kidney cells	79
2016	Bone-marrow stromal cells derived exosomes secretion of miR-145	Neurorestorative effects in T1D stroke rats	82
2016	Human endothelial progenitor exosomes	Acceleration cutaneous wound healing in diabetes	108, 109
2016	Mesenchymal stem cell derived exosomes as anti-miR-375 carriers	Improve islet transplantation	83
2016	Bone marrow-derived exosomes	Repairing diabetes-induced damaged neurons and astrocytes	110
2016	Hsp20-engineered exosomes	Therapeutic agent for diabetic cardiomyopathy	111
2016	miR-let7-engineered MSC exosomes	Attenuate renal fibrosis in T1D	112
2016	Urine-derived stem cells	Prevention of kidney complications in T1D	79
2015	Murine pancreatic ß-cell line exosomes	Differentiation of bone marrow cells into insulin-producing cells in the subcutaneous Matrigel platforms	85
2015	Fibrocyte derived EXOs release hsp90α, total and activated signal transducer and activator of transcription 3, proangiogenic (miR-126, miR-130a, miR-132) and anti-inflammatory (miR124a, miR-125b) microRNAs, and a microRNA regulating collagen deposition (miR-21).	Treatment of diabetic ulcers	113

for T1D are invasive organ transplant procedures, only available for the most severe cases of T1D due to significant complications associated with the procedure. Nevertheless, clinical islet cell transplantation has emerged as a less invasive therapeutic alternative to whole pancreas transplant for the treatment of the most severe cases of T1D. In combination with the islet transplantation setting, exosomes may represent an exciting new therapy not only for improvement of islet function[83], but also for the induction of transplant tolerance[81] and as alternative source to obtain insulin-producing ß-cells as previously shown[85].

Conclusions

EXOs provide a potential source of cell or tissue type specific biomarkers for the diagnosis, treatment and evaluation of disease progression of T1D. Further characterization of EXOs, their mechanisms of action and their relationship with different stages of disease will provide the basis for a better understanding of the role of EXOs in the pathogenesis of T1D and their possible use as therapeutic tools.

Acknowledgements:
This work was supported by the Diabetes Research Institute Foundation, Miami, FL, USA.

Declaration of interest:
The authors declare no competing financial interests.

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