

Microvesicles/exosomes as potential novel biomarkers of metabolic diseases

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Abstract: Biomarkers are of tremendous importance for the prediction, diagnosis, and observation of the therapeutic success of common complex multifactorial metabolic diseases, such as type II diabetes and obesity. However, the predictive power of the traditional biomarkers used (eg, plasma metabolites and cytokines, body parameters) is apparently not sufficient for reliable monitoring of stage-dependent pathogenesis starting with the healthy state via its initiation and development to the established disease and further progression to late clinical outcomes. Moreover, the elucidation of putative considerable differences in the underlying pathogenetic pathways (eg, related to cellular/tissue origin, epigenetic and environmental effects) within the patient population and, consequently, the differentiation between individual options for disease prevention and therapy – hallmarks of personalized medicine – plays only a minor role in the traditional biomarker concept of metabolic diseases. In contrast, multidimensional and interdependent patterns of genetic, epigenetic, and phenotypic markers presumably will add a novel quality to predictive values, provided they can be followed routinely along the complete individual disease pathway with sufficient precision. These requirements may be fulfilled by small membrane vesicles, which are so-called exosomes and microvesicles (EMVs) that are released via two distinct molecular mechanisms from a wide variety of tissue and blood cells into the circulation in response to normal and stress/pathogenic conditions and are equipped with a multitude of transmembrane, soluble and glycosylphosphatidylinositol-anchored proteins, mRNAs, and microRNAs. Based on the currently available data, EMVs seem to reflect the diverse functional and dysfunctional states of the releasing cells and tissues along the complete individual pathogenetic pathways underlying metabolic diseases. A critical step in further validation of EMVs as biomarkers will rely on the identification of unequivocal correlations between critical disease states and specific EMV signatures, which in future may be determined in rapid and convenient fashion using nanoparticle-driven biosensors.

Keywords: adipose tissue, epigenetics, glycosylphosphatidylinositol, microparticles, microRNA, type II diabetes

Biomarkers Definitions

Under the direction of the National Institute for Health (NIH), the Biomarkers and Surrogate Endpoint Working Group has agreed on key definitions as well as a classification system for biomarkers as follows: (1) a biomarker represents “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention”; (2) a clinical endpoint represents “a characteristic

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or variable that reflects how a patient feels, functions, or survives”; and (3) a surrogate endpoint represents “a biomarker intended to substitute for a clinical endpoint with the potential for predicting the clinical benefit or harm (or lack of benefit or lack of harm) on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence.”^{1,2} Thus, the very aims of the use of biomarkers, clinical endpoints, and surrogate endpoints are: (1) the improvement of the prediction, diagnosis and prognosis, particularly of common complex multifactorial diseases; and (2) the facilitation of the drug discovery and development processes.^{3,4}

According to the European Medicine Agency (EMA), clinical endpoints are distinct measurements or analyses of disease characteristics observed in a study or clinical trial that reflect the effect of the therapeutic intervention.⁵ Consequently, clinical endpoints are regarded as the most reliable indicators for disease or therapeutic responses. The definition of the US Food and Drug Administration (FDA) is much more practical and focused, stating that surrogate endpoints or biomarkers are “used in clinical trials as a substitute for a clinically meaningful endpoint, represents a direct measure of how a patient feels, functions or survives, and is expected to predict the effect of the therapy.”⁶

In other words, a biomarker is an indicator of change and therefore fluctuates as a function of time and biological influence. Importantly, this strict definition of a biomarker excludes single nucleotide polymorphisms. Lastly, from the perspective of the pharmaceutical industry, a pragmatic

definition of the term biomarker describes it as: “A measurable property that reflects the mechanism of action of the molecule based on its pharmacology [and] the pathophysiology of the disease, and [may] be useful for internal decision-making within a pharmaceutical company.”⁷

Moreover, a general classification system has been introduced for biomarkers that discriminates type 0 biomarkers, which measure the natural history of a disease and correlate over time with established clinical indicators; type I biomarkers, which indicate the intervention effect of a drug; and type II biomarkers, which are considered as surrogate endpoint biomarkers (Figure 1). Both type I and type II biomarkers are ultimately aimed at monitoring the so-called wellness index, which ranges from 0 (death) to 100 (perfect homeostatic control under healthy conditions). As a function of age, environment, and genes, individuals strive to maintain a biological equilibrium of homeostatic control. Various biomarkers can be used to determine the personalized progression from homeostasis via disease initiation, disease development, and further disease progression to final disease outcome (Figure 1).⁸

Applications

Importantly, in most cases, the biomarkers for a given indication are used primarily in the pattern recognition mode on the basis of a set of unidentified markers that may be genomic, transcriptomic, proteomic, lipidomic, metabolomic, or a combination of datasets.^{9,10} A priori application of these type I-like biomarkers does not necessitate the determination

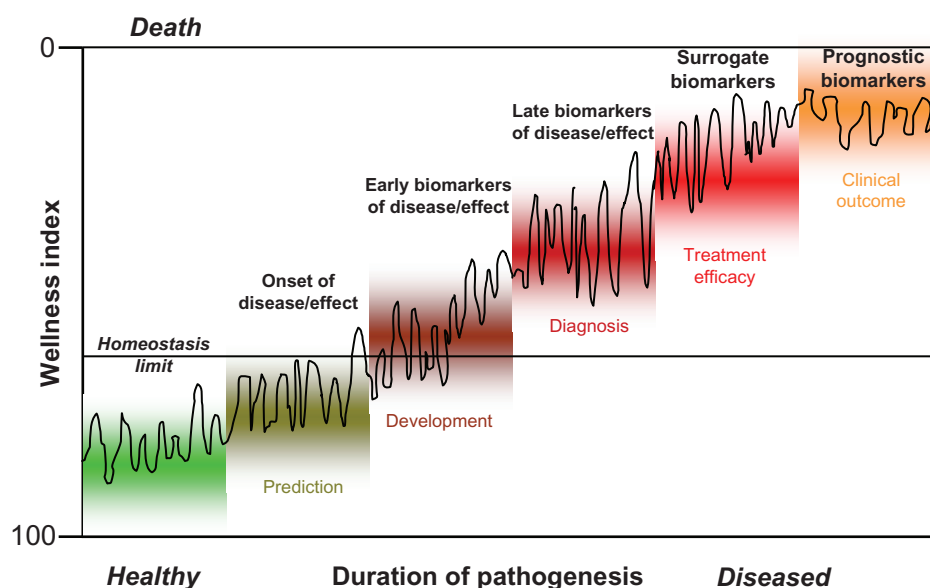


Figure 1 The role of biomarkers in following the pathogenesis of individuals on basis of the wellness index (see text for details).

of the biomarker constituent identities since the pattern or signature alone denotes the specific biological activity.¹¹ In this regard, EMVs (see “EMVs as biomarkers: General considerations”) belong to type I biomarkers. In addition, the use of type II-like biomarkers (transcripts, proteins, metabolites) for deciphering or screening the pathogenetic mechanism, prediction, diagnosis, and monitoring of common complex diseases is of critical importance and requires their structural identification and validation (for at least one of them). This approach may be supported or even substituted by the discovery of complex biomarker panels of type 0 to monitor specific disease states along the complete pathogenetic pathway for prediction, initiation, development, diagnosis, progression, regression, and treatment efficacy of the disease (Figure 1).^{1,11–15} The expectation of a good biomarker ranges from a molecular signature of structurally unidentified markers to a complete panel of identified biomarkers specific to the pathogenesis being studied. Importantly, the work required to establish the reliability and validity of a new biomarker should not be underestimated; it needs detailed planning for each combination of a clinical indication and a mechanism of action.¹⁶ For example, type 0 biomarkers can be validated longitudinally in a well-defined patient population against a gold standard clinical assessor. In contrast, type I biomarkers should be validated in parallel with the drug candidate, and type II biomarkers must be relevant both to the mechanism of action of the drug and to the pathophysiology of the disease.

Critical questions for the discovery and development of modern drugs and the adequate use of biomarkers are how to adequately transform data into information and thence into knowledge and how to apply that to both processes. The general hope is that biomarker data will provide more predictive information and knowledge about the alterations in the biological processes induced after administration of the drug,¹⁷ which should allow better predictive capability and decision-making on the part of scientists and managers involved in the drug-finding procedure.¹⁸

Biomarkers for metabolic diseases Pathogenesis

The pathogenetic mechanisms underlying metabolic diseases have previously been assumed to rely on abnormal triacylglycerol storage, which is driven by excess of energy intake and insufficient energy expenditure in the case of development of metabolic syndrome, insulin resistance, and obesity, and accompanied or followed by considerable reduction in the number and function of pancreatic β -cells

in the case of development of type II diabetes.^{19–21} Chronic low-grade inflammation^{22,23} and oxidative stress^{24,25} are regarded as causally involved in both the induction and the progression of metabolic diseases. Importantly, the levels of a subset of proinflammatory adipokines, such as interleukin-2/6 (IL-2/6), tumor necrosis factor- α (TNF α), adiponectin, ferritin, and C-reactive protein (CRP) have been demonstrated by epidemiological studies to be positively correlated to the degree of insulin resistance, impaired glucose tolerance and homeostasis, and excess of adipose tissue mass.^{26,27} They have thus been regarded as the most predictive biomarkers for metabolic diseases.^{28–30} The same is true for oxidized low-density lipoprotein (LDL), which induces the monocytes in plasma for their movement into and residence in the adipose tissue upon binding to the walls of the constituting vascular endothelial cells. The elevated infiltration of the adipose tissue with immune cells seems to function as the driver for the emergence of defects in insulin signaling, ie, insulin resistance; increase in adipocyte number and size, ie, obesity; and plaque destabilization and rupture, ie, atherogenesis.^{31–33} Moreover, elevated oxidative stress as well as the redox state in the adipose tissue has also been associated with the early phases in the pathogenesis of metabolic diseases.³⁴ Interestingly, both inflammation and oxidative stress have consequences for insulin signaling, adipocyte proliferation and differentiation, and adipocyte apoptosis and angiogenesis, ultimately resulting in type II diabetes. Obesity and atherosclerosis have been linked to specific expression patterns of miRNAs (see “EMVs as biomarkers: Obesity”).^{35–37}

Currently used biomarkers

Currently, the prediction within a limited period during the development of a metabolic disease prior to its diagnosis (typically 5–10 years for type II diabetes), as well as its definite diagnosis and prognosis of the future disease outcome, are based on the measurement of the levels of typical and easily accessible serum parameters, such as carbohydrate and lipid metabolites (eg, glucose, triacylglycerol, cholesterol, lipoproteins), small molecule intermediates (eg, 2-hydroxybutyrate) and metabolites (eg, creatinine) as well as surrogate entities (eg, glycated hemoglobin HbA_{1c}).^{38–42} In routine clinical practice, these so-called traditional biomarkers in combination enable the prediction of type II diabetes with a probability of about 0.65 to 0.75 (with 0.5 representing chance in throwing a dice) (Figure 2). The supplementation with information about physical body parameters (BMI, waist-to-hip ratio, sex)

as well as the health state and lifestyle of the probands (eg, blood pressure, smoking, fitness, food consumption as evaluated in the Deutsche Risikoabschätzung⁴³) leads to a further increase in the probability of prediction to about 0.85 or 0.90.⁴³ In future, functional assays, currently applied on a routine basis only for the confirmatory diagnosis of a metabolic disease, such as glucose and insulin tolerance tests, as well as euglycemic clamp studies for type II diabetes and noninvasive imaging procedures⁴⁴ currently in the stage of clinical testing and approval, such as magnetic resonance imaging (MRI) of the β -cell mass for type II diabetes and positron emission tomography (PET) of the brown adipose tissue mass for obesity, will supplement the portfolio of traditional biomarkers and further improve their predictive power (Figure 2). However, in any case, the prerequisite and, therefore, the critical disadvantage of the traditional, functional, and imaging biomarkers is that the earlier the time points are envisaged for the prediction, potential prevention, and therapy, the less informative they are. On the other hand, they are of particular value for prediction along further disease development and advanced stages of the pathogenesis. In sum, therefore, these phenotypic biomarkers do support prediction independent of the individual life stage and style in contrast to the prediction of disease susceptibility by genotypic biomarkers (Figure 2).

In research clinical studies, the determination of so-called novel biomarkers, predominantly peptides

and proteins, such as hormones, cytokines (eg, TNF- α), adipokines (leptin, adiponectin), incretins (GLP-1), and others (eg, ferritin, cross-reactive protein), in combination but not alone, led to prediction values approaching, but hardly exceeding, those achieved with well-designed combinations of traditional biomarkers.⁴⁵ The highest prediction probabilities have been reported for combinations of traditional and novel biomarkers,⁴⁶ as for example those evaluated by the “EASD Risk Score”.⁴⁶ However, because of the partial overlap of the pathogenic mechanisms and pathways covered by the two classes of biomarkers, the predictive values of the combinations did not increase to a degree as calculated from the individual contributions of each of the two classes.

In contrast to phenotypic biomarkers, genotypic biomarkers, ie, determination of the complete genetic profile encompassing all relevant polymorphisms in all metabolic disease susceptibility genes, would fulfill the typical demand for life stage/style-independent prediction. However, the research clinical studies so far reported were rather disappointing: predictive values for type II diabetes ranging from 0.54 for genetic polymorphisms in three independent susceptibility genes in combination²⁸ to 0.60 for single nucleotide polymorphisms in 18 distinct susceptibility genes (Figure 2).^{29,43,45} Apparently, common complex multifactorial metabolic diseases cannot be predicted with sufficient probability or precision on the basis of genotypic biomarkers

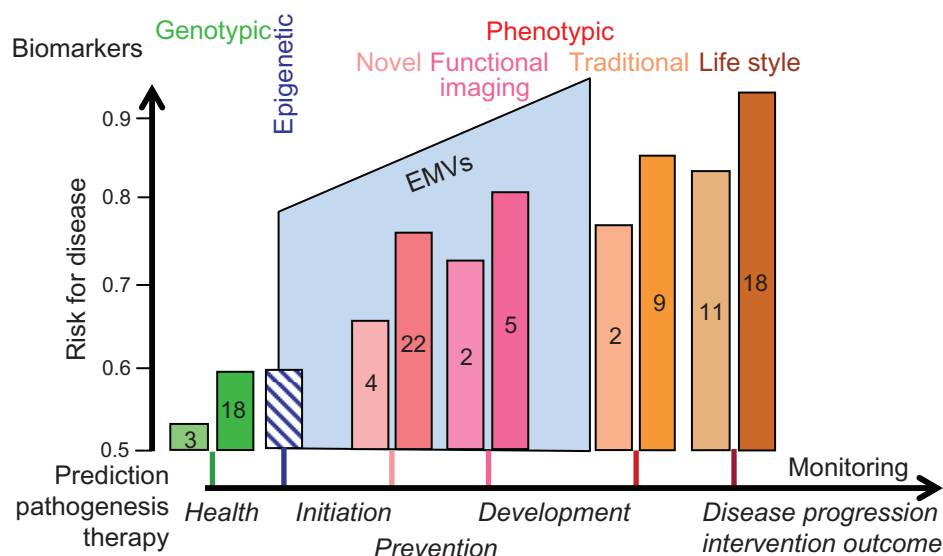


Figure 2 Correlation between the prediction of the risk for disease and the earliest time point feasible for its prediction.

Notes: The risks for disease are given for the different stages during the pathogenesis based on genetic biomarkers (in green), epigenetic biomarkers (in hatched blue), novel/functional biomarkers (in pink), and traditional/life style biomarkers (in brown) in comparison (hypothetical) to EMV signatures as biomarkers (in blue). For each type of biomarker, the number of biomarker members measured and combined for prediction is indicated within the bars.

Abbreviation: EMVs, exosomes and microvesicles.

that have been derived from the identification of disease genes as well as susceptibility genes altered in their amount and/or function by mutations, single nucleotide polymorphisms, copy number variants, monoallelic expression, and complex combinations. Even more discouraging was the repeated observation that combining the polymorphisms identified in known metabolic disease susceptibility genes with traditional and novel biomarkers only marginally increased the total predictive value compared to the values of the traditional and/or novel biomarkers in the absence of genotypic markers.^{27–29} It may be argued that in future the further increase in the number of susceptibility genes identified for metabolic disorders will lead to considerable improvement of the predictive power of genotypic biomarkers in polymorphism and gene combinations of increasing complexity, affecting multiple target tissues and pathogenic pathways. However, the path from the genotype to the phenotype with underlying gene–gene interactions, gene–environment interactions, genome plasticity (somatic and mitochondrial mutations), and epigenetic modifications is long and complex. For these principal considerations, which are beyond the scope of this review article, it remains questionable whether the complete genetic profiling per se, ie, the determination of all relevant (single nucleotide) polymorphisms of each susceptibility gene in all relevant combinations encompassing each complete disease mechanism (eg, insulin release and insulin signaling) in all relevant tissues contributing to metabolic diseases (eg, β -cells and liver, muscle, adipose tissues) will truly enable predictions of metabolic diseases with the required high probabilities of ≥ 0.90 .

Thus, there is a critical gap between the genotypic biomarkers with their capability for very early and life stage-independent prediction of rather limited probability and the traditional as well as functional/imaging biomarkers with

their capability for rather late and pathogenesis-dependent prediction of higher probability (Figure 2). Apparently, this gap has not yet been filled by novel biomarkers with their intermediary predictive values and intermediary positioning between the initiation of the pathogenesis and the full development of a complex common metabolic disease (Figure 2). With regard to drug discovery and development, the efficacy of new antidiabetic drugs has been evaluated traditionally in clinical trials using morbidity and mortality as the endpoints (Figure 3). However, such trials may require 10,000–15,000 subjects and at least 5 years of follow-up to demonstrate significant benefits. Smaller and shorter studies based on biomarkers and surrogate endpoint effects for monitoring the various stages from drug discovery via drug development to drug approval have the potential to revolutionize the drug finding and approval process (Figure 3).

Requirements for future biomarkers

In future, biomarkers should enable monitoring of the upregulation of the number and size of adipocytes, the downregulation of the number and/or functionality of pancreatic β -cells, the impairment in insulin sensitivity of peripheral tissues, such as muscle, fat, and liver, the development of impaired glucose tolerance and homeostasis, and finally the manifestation of obesity and type II diabetes and further progression to late complications of diabetes, such as retinopathy, nephropathy, neuropathy, and micro/macroangiopathy. These biomarkers should preferably circulate in the plasma and allow monitoring (1) of the overall pathogenesis or critical pathogenic steps; (2) of drug efficacy in a target-independent fashion; and (3) with limited expenditure in preclinical and clinical studies. Furthermore, they should fill the above-mentioned gap left by genotypic, traditional, and novel biomarkers (Figure 2).

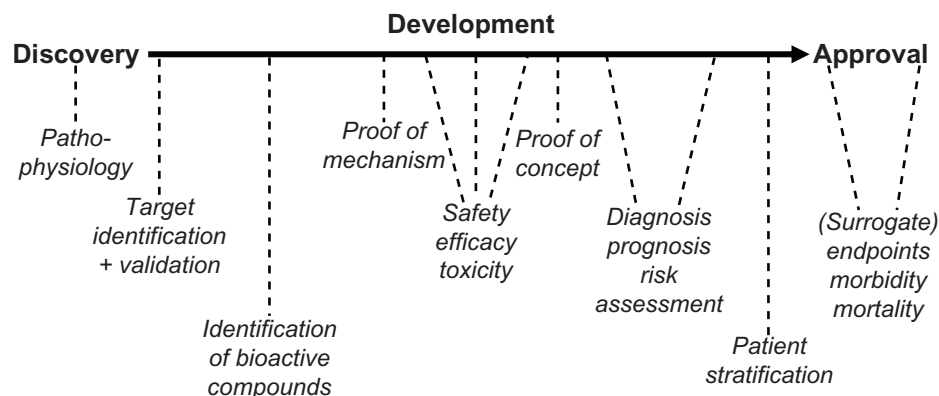


Figure 3 Paradigm for the use of biomarkers in drug discovery, development and approval.

Although it seems reasonable to determine biomarkers in disease-relevant and affected organs, tissues and cells where they typically occur at higher concentrations, it is of huge practical advantage to measure them in the plasma. However, the huge dynamic range in the amounts of the individual protein components in plasma (>10 orders of magnitude in difference) hampers the discovery as well as routine determination of novel biomarkers, since typical plasma proteomics is biased towards the detection of primarily high-abundance proteins. This necessitates complex and tedious fractionation procedures that may facilitate the access to low-abundance proteins. However, these are not typically compatible with high-throughput analysis, which is, however, a prerequisite for the monitoring of large clinical trials that are appropriate and required for metabolic diseases.

Exosomes and microvesicles (EMVs)

In contrast, plasma proteomics based on EMVs have the distinct advantage that the information obtained may encompass a considerable reservoir of novel biomarker candidates being transferred from organs, tissues, and cells to that compartment and protected from degradation. Moreover, the plasma EMV sub-proteome is characterized by a smaller dynamic range and a higher portion of undegraded soluble and membrane proteins at considerably high concentrations, and their amount and composition is determined by specific stimuli (eg, drugs) or microenvironmental and pathogenic factors (eg, cellular stress, high glucose). EMVs are expected to fulfill and connect the above criteria of very early prediction, high prediction probability, and feasibility of measurement. This expectation is based on (1) the structure and composition of EMVs with constituent mRNAs encoding genotypic biomarkers as well as constituent cytokine biomarkers, signaling proteins, receptors, transporters, and enzymes; (2) the function of EMVs in intercellular information transfer in various pathogenic processes; (3) the sensitivity of EMVs toward environmental stimuli with regard to their release from the donor cells of almost each tissue and organ into the circulation; and (4) the accessibility and ease of detection and technological determination of EMVs in the plasma. For routine applications in the mid-term, it will be tremendously important to identify significant and physiologically relevant correlations between EMVs and metabolic diseases along subsequent stages of each of the different disease mechanisms covering (1) the healthy state; (2) the initiation of the disease process; (3) its subsequent development to the established disease; (4) the benefits and failures of therapeutic intervention; and (5) the further progression to complications linked to diabetes and obesity (Figure 2).

Structure and composition

It has long been known that small membrane vesicles are released from most animal cell types,^{47,48} such as mast cells,⁴⁹ dendritic cells, B lymphocyte cell lines,⁴⁹ astrocytes, platelets, neurons, endothelial cells, and epithelial cells.^{50,51} They have been regarded as “extracellular organelles” and a family member of the “bioactive vesicles” (Figure 4). They have the same topology regarding outer and inner phospholipid bilayer leaflets as the donor cell, and are of variable diameters, which are used to discriminate the larger so-called microvesicles (200–1000 nm) and the smaller so-called exosomes (50–200 nm) (Figure 4). It is critically important to distinguish EMVs from the other large membrane vesicles (0.5–3 μm), the so-called apoptotic bodies, which are released from almost each cell type when they are challenged by apoptotic and death signals or mechanical stress (Figure 5).⁵² Despite a variety of morphological and structural similarities, apoptotic bodies and EMVs appear to differ considerably with regard to the cellular origin and molecular composition as well as the releasing signals and mechanisms. Interestingly, EMVs were identified more than three decades ago as being released from reticulocytes during their maturation into erythrocytes, whereby the transferrin receptor as a constituent component of those EMVs becomes downregulated in the mature erythrocytes.⁵³

EMVs have been detected in the circulatory system (plasma) and various body fluids, such as urine, bronchoalveolar lavage, mucus, saliva, bile, ascites, cerebrospinal fluid, and breast milk.^{54–56} On the basis of the identification of tumor-derived EMVs in the plasma of patients with glioblastoma, multiform malignant glioma, ovarian carcinoma, and lung adenocarcinoma,^{55,57} it may be concluded that EMVs are transferred across the blood-brain barrier and are released from both the apical and basolateral plasma membranes of polarized epithelial and endothelial cells, respectively. EMVs harbor a wide variety of (glyco)phospholipids, mRNAs, and microRNAs (miRNAs), but not ribosomal RNAs,⁵⁸ in concert with soluble, peripheral, transmembrane, and glycosylphosphatidylinositol- (GPI)-anchored proteins (Figures 4 and 5) with overlapping yet distinct patterns between different cell types (Tables 1–4). It seems plausible that their composition depends on the cell type from which they originate and is therefore heterogeneous, which is revealed by proteomic analysis of EMV components in the large scale derived from various cell types⁵⁹ (detailed summarized information in <http://dir.nhlbi.nih.gov/papers/lkem/exosome>) and from the urine.⁶⁰ The latter study described the presence of 1132 polypeptides and, in addition, of phosphoproteins in EMVs.

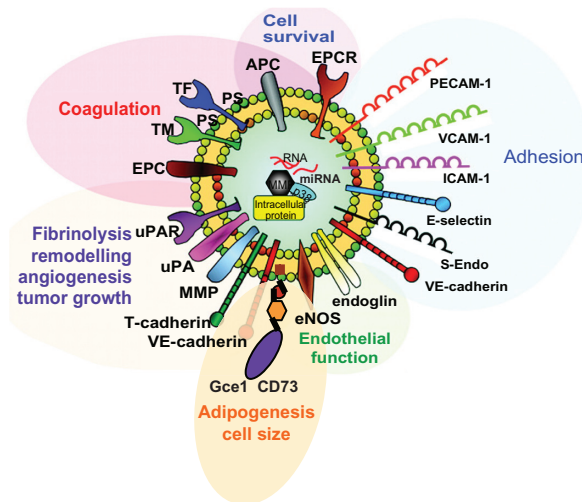


Figure 4 Structure of "model" EMVs with functions of some of their components (see text for details).

Note: © 2011 Wolters Kluwer Health. Adapted with permission from Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol.* 2011;31(1):27–33.³⁷⁶

Abbreviations: EPCR, endothelial protein C receptor; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; S-Endo, CD146/melanoma cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloproteinases; uPA, urokinase plasminogen activator; uPAR, uPA receptor; EPC, endothelial protein C; TM, thrombomodulin.

Moreover, the current version (3.1) of ExoCarta⁶¹ compiles 11,261 protein entries, 2375 mRNA entries, and 764 miRNA entries derived from 134 studies that deal with EMVs.⁶¹ All these EMV components originate from the cytoplasm,

nucleus, cytoskeleton, proteasome, plasma membranes, and intracellular membranes (eg, mitochondria, endoplasmic reticulum) of the donor cell (Figure 5). Thus, the differential composition of EMVs from various body fluids is a prerequisite for their potential use as biomarkers for metabolic diseases.

Biogenesis

At present, the detailed mechanisms underlying the biogenesis of EMVs remain unclear. According to current models, microvesicles develop as buds directly at the plasma membrane and are subsequently released by shedding under control of the intracellular calcium concentration and reorganization of the cytoskeleton, with annexin playing a key regulatory role (Figure 6A). Alternatively, the membrane of intracellular endosomes buds into the luminal space. After shedding, they form multivesicular bodies (MVB), which in the course of trafficking to and fusion with the plasma membrane result in exosomes released into the extracellular space (Figure 6B).^{47,62} The energy-dependent formation of soluble NSF attachment protein receptor (SNARE) complexes⁶³ is thought to mediate the attachment of the exosomal membranes (via v-SNAREs) with the target plasma membranes (via t-SNAREs).⁶⁴ Upon initiation of complex assembly at the amino-terminal regions of the SNAREs and further movement toward the membrane-anchoring carboxy-terminal

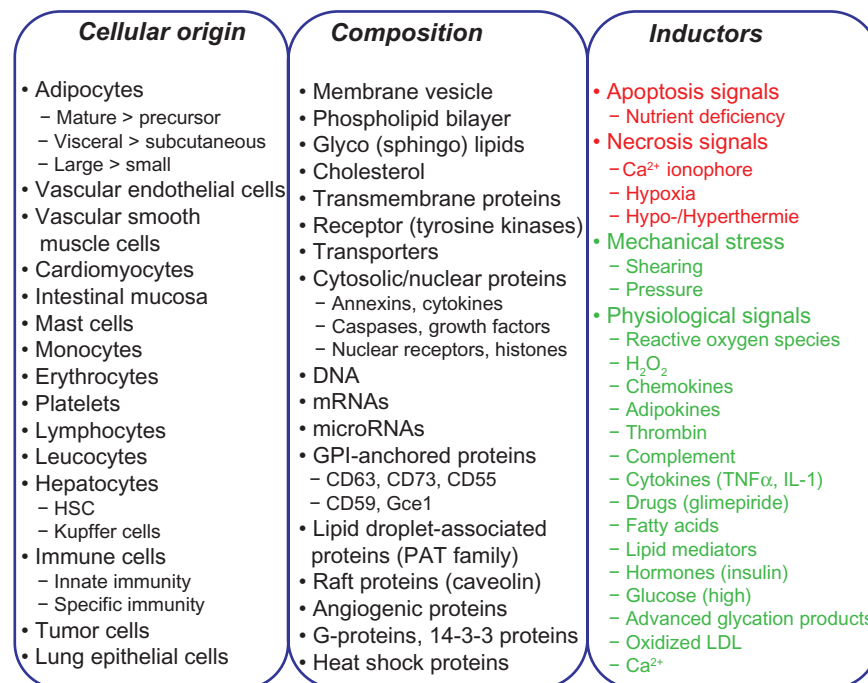


Figure 5 Cellular origin and composition of EMVs and apoptotic vesicles released from donor cells in response to inductors, such as physiological and stress signals (in green) or apoptosis and necrosis signals (in red).

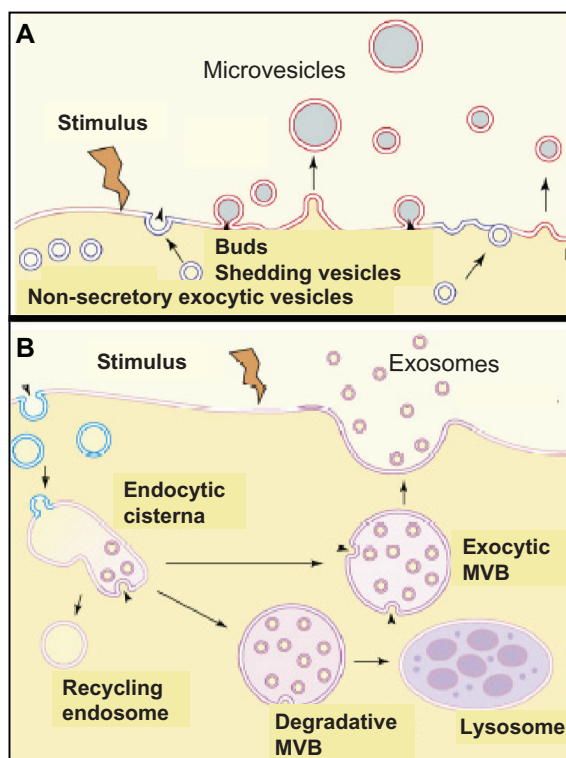


Figure 6 Molecular mechanisms for the release of exosomes. **(A)** involves the budding and subsequent shedding of specialized areas of the plasma membrane, which are identical to lipid rafts (in red), from the donor cells. **(B)** involves exocytosis, ie, fusion with the plasma membrane, of exocytic multivesicular bodies (MVB) which originated from endocytic cisternae and escaped degradation by degradative MVB and lysosomes as well as recycling by endosomes, and of microvesicles.

Note: These vesicles are structurally and functionally distinct from nonsecretory exocytic vesicles. © 2008, Elsevier. Adapted with permission from Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2008;19:43–51.⁴⁷

regions,⁶⁵ an intermediary complex constituted by SNAP-25 and syntaxin-1 is formed that interacts with synaptobrevin-2. The SNARE-dependent docking of the MVB at the plasma membrane is apparently regulated by Rab27a, Rab27b, and Rab35^{66,67} since downregulation of their expression and/or activity led to size increases/accumulation and redistribution to the perinuclear region of the MVB/endosomes.

A recent study described a novel and interesting mechanism for the regulation of exosome biogenesis and release, which involves members of the tetraspanin protein family. The tetraspanin transmembrane proteins possess four membrane-spanning domains and are engaged in a very wide range of specific molecular interactions that result in the formation in the plane of the membrane of tetraspanin-enriched microdomains, which are identical or similar to lipid rafts.^{68–71} Tetraspanins have been implicated in a multitude of biological processes, such as cell adhesion, migration, fusion, and signal transduction through their associated partner molecules.^{72,73} Microarray

analysis showed that CD9 expression in tumor cells correlated with the down-regulation of several Wnt family genes and their targets, suggesting that CD9 may act as an upstream negative regulator and tumor suppressor in the Wnt signaling pathway.⁷⁴ Previous immunoelectron microscopic studies showed that several members of the tetraspanin family, including CD63 and CD82, are rich in exosomes.⁷⁵ At steady state, tetraspanins CD9 and CD82 are organized in a signaling complex with the membrane adaptor and β -catenin-binding protein, E-cadherin, at the plasma membrane.⁷⁶ This signaling complex, including CD9, CD82, E-cadherin, and the transcription factor, β -catenin, becomes internalized and delivered to early endosomes where the exosome biogenesis is initiated with inward vesicle budding. After maturation of the exosome-containing endosomes into late MVB, they fuse with the plasma membrane and thereby release the β -catenin-harboring exosomes. Thus, in general, the tetraspanins CD9/CD82 seem to drive and control the release of exosomes. In the case of β -catenin-containing exosomes they apparently trigger the reduction in the cytoplasmic/nuclear pool of β -catenin with the accompanying downregulation of Wnt/ β -catenin signaling and tumorigenesis.⁷⁶

According to their biogenesis, a principal differentiation between microvesicles and exosomes seems feasible (Figure 6; Table 5); however, their classification according to composition and cellular origin is problematic (Figure 5). This is predominantly due to the lack of unambiguous physical properties or unique molecular markers of EMVs (Tables 1–4) and, consequently, the difficulties in knowing their cellular origin and biogenetic pathway. Moreover, a number of experimental observations are difficult to reconcile with a strict separation between microvesicle and exosome biogenesis (Table 5). For instance, (glyco)sphingolipids and GPI-anchored proteins – eg, the prion protein⁷⁷ – already embedded in the outer leaflet of the plasma membrane can also be efficiently released via exosomes.⁷⁸ This process can be quite extensive, as demonstrated in reticulocytes, which release approximately 50% of GPI-anchored acetylcholinesterase from the plasma membrane via exosomes during their differentiation into erythrocytes.⁷⁹ Thus, exosomal proteins and phospholipids are able to bud directly from the plasma membranes of the donor cells.⁸⁰ In addition, exosomes are also detectable as deep invaginations of plasma membranes and thus possibly share the site of biogenesis with that of microvesicles.⁸¹ Consequently, it has been speculated that exosomes and microvesicles use similar or overlapping pathways in their biogenesis. Surprisingly, recent findings

Table I Some protein components identified in EMVs from rodent adipocytes^{300,302,307,322}

Protein class	Name	Accession number	Size (kDa)
Metabolic enzymes	Phosphoglycerate kinase I	P09411	44.5
	NADP-dependent malic enzyme	XP923436	61.5
	Citrate synthase	AAH13554	51.7
	Malate dehydrogenase	CAA30274	35.6
	Fatty acid binding protein-3	BAE24952	14.8
Secretory/membrane proteins	Fatty acid binding protein-4	CAAJ18597	14.6
	MFG-E8	BAE42274	51.2
	Adiponectin	BAC30866	26.7
	Fasting-induced adipose factor	AAF86342	45.5
	CD9	AAH70474	25.2
	CD63	XP_919469	25.7
	Integral membrane protein TAPA-I	AAB19417	25.8
	Clathrin heavy chain	BAE25402	114.1
	MMP-2	AAH70430	74.0
	MMP-9	XP_925466	98.5
	Coagulation factor II	AAH13662	70.2
	Coagulation factor V	BAE23393	115.7
	Fibulin 2	AAD34456	131.9
	Annexin II	NP_067449	49.4
	Complement C3	P01027	186.4
Heat shock proteins	Hsp I	AAI06113	59.4
	HSP60	XP_925296	21.2
	HSP70	XP_930845	70.9
	HSP75	BAE39316	80.1
	HSP84	PI1499	83.3
	TCP-I chaperone family β -subunit	BAA95054	52.4
	TCP-I chaperone family γ -subunit	BAE42283	31.5
	TCP-I chaperone family ϵ -subunit	BAC97866	59.7
GPI-anchored proteins	Gce I		54.2
	CD73		73.5

Abbreviation: EMVs, exosomes and microvesicles.

suggested that exosome biogenesis does not depend on the formation of MVB since the latter but not the former process requires functional endosomal sorting complexes required for transport (ESCRT) machinery.^{80,82} This argues for identical MVB-independent biogenetic pathways for both microvesicles and exosomes. Given these uncertainties, it has recently been suggested to use the collective acronym, EMVs, for exosomes plus microvesicles⁸³ for any released nonapoptotic small membrane vesicle (Figures 5 and 6) that is also used in this review article.

Releasing signals

Multiple signaling mechanisms are presumably involved in the cell- or agonist-specific release of both types of EMVs (Figure 5). It is important to point out that EMV release is not a random process, such as the degradation of plasma membranes of dying necrotic cells, but a highly controlled process triggered by a multitude of structurally diverse stimuli (Figure 5). For instance, in platelets, EMV release is induced by the following stimuli with increasing potency:

epinephrine, adenosine diphosphate, thrombin, collagen, thrombin plus collagen, and Ca^{2+} ionophore A23187.⁸⁴ Furthermore, the complement membrane attack complex C5b-9 or antiplatelet antibodies represent other stimuli that cause EMV release in platelets. In addition to biological and chemical stimuli, mechanical factors, such as shear stress, induce EMV release in platelets and many other cell types. Significantly, many stimuli can trigger the release in additive or even synergistic fashion to other stimuli.

miRNA components

miRNAs are small, noncoding RNA sequences of about 22 nucleotides⁸⁵ expressed in animals, plants, and viruses.⁸⁶ All precursor miRNAs have stem loop structures that are cleaved by the Drosha and Dicer protein complexes to form mature functional miRNA. Through the formation of RNA-induced silencing complexes, miRNAs can either cleave mature mRNA molecules or inhibit their translation, thus representing an additional posttranscriptional layer of gene regulation. Interestingly, miRNAs were found

Table 2 Some protein components identified in EMVs from rodent adipocytes^{300,302,307,322}

Protein class	Name	Accession number	Size (kDa)
Ubiquitin/proteasome-related proteins	26S proteasome subunit α 1	BAE40184	20.2
	26S proteasome subunit β 3	XP_920004	18.8
	26S proteasome subunit β 5	O55234	22.9
	26S proteasome subunit Rpn13	BAE42495	38.2
	Ubiquitin-activating enzyme E1	BAE25369	117.8
Nuclear proteins	Histone H1.1	NP_034507	21.6
	Histone H1.5	BAB32001	22.3
	Histone H1.3	BAE33250	22.1
	Histone H2A	AAH10564	14.9
	Histone H2B	XP_214483	16.6
	Histone H4	XP_911558	18.6
	40S ribosomal protein SA	XP_909595	9.0
	40S ribosomal protein S8	XP_890310	37.6
	60S ribosomal protein L7	CAA41028	17.7
	60S ribosomal protein L18a	BAE40206	20.6
Cytoskeleton	Actr1b protein	AAH24861	19.6
	β -Actin	BAB40317	15.0
	Tubulin α 2	XP_89880	28.1
	Tubulin β 2	XP_917827	49.8
	Tubulin β 5	BAE30573	42.1
Membrane transport/fusion	Annexin II	XP_622573	32.6
	Annexin V	BAE31952	35.7
Miscellaneous	Galectin I	AAH02063	14.9
	Galectin 3	BAE31013	64.5
	Translation elongation factor-1	XP_921527	19.6
	Translation elongation factor-2	BAC28120	95.2
	Translation initiation factor 4A	XP_929093	37.4
	Major vault protein	AAL02325	95.9
	I4-3- β	AAC14343	28.1
	Dynein heavy chain	NP_084514	531.7
	Albumin	BAC34145	68.7

Abbreviation: EMVs, exosomes and microvesicles.

incorporated into EMVs released from primary or cultured cells in vitro or circulating in the plasma. For instance, a large number of miRNA species has been detected associated with EMVs in the plasma of normal subjects, which are predicted to control homeostasis and metabolism of hematopoietic cells.⁸⁷ Importantly, the miRNA species (as revealed by advanced array technologies) of those EMVs did not reflect the total miRNA profiles of the donor cells,^{57,58,87–89} which is an argument for rather selective miRNA packaging into EMVs. Nevertheless, EMVs released from T-cells, B-cells, and dendritic cells have been demonstrated to harbor miRNA species unique to the expression pattern in their donor cells.⁹⁰ The molecular mechanisms underlying the selective packaging of miRNAs into EMVs remain unknown so far.

Importantly, miRNAs have been demonstrated to be more stable than their cellular counterparts⁹¹ and to resist degradation during prolonged storage and repeated freezing/thawing cycles.⁹² This apparent stability of EMV contents

underscores the attractiveness of EMVs as biomarkers. Moreover, during the development of many diseases and in a multitude of pathological states, EMVs prepared from patients were found to harbor specific miRNAs that were detected at lower levels or not at all in EMVs from normal healthy subjects.^{57,88,93,94} For instance, levels of miR-133a, which regulates the NFATc4 protein known to accompany or even induce the development of cardiac hypertrophy, have been found increased in circulating EMVs isolated from injured myocardium of patients with cardiometabolic diseases.⁹⁵ In agreement, the administration of miR-133a antagomirs that specifically inhibit miR-133a function was reported to decrease the extent of cardiac hypertrophy.⁹⁵

The administration and targeted cellular delivery of specific miRNAs via EMV-like vehicles prepared from natural sources and subsequently chemically modified or reconstituted in vitro is under intense investigation and

Table 3 Some protein components identified in EMVs from various cell types^{303,322,361,363,372–375}

Protein class	Name	Cell type
Antigen presentation	MHC class I and II	B-cells
		Dendritic cells
		Enterocytes
		Tumor cells
		T-cells
Integrins	alpha4β1 alphaMβ2 β2 alphaLβ2	Mastocytes
		Reticulocytes
		Dendritic cells
		T-cells
		Mastocytes
Immunoglobulin-family members	ICAM1/CD54	B-cells
		Dendritic cells
		Mastocytes
		P-selectin
		A33 antigen
Cell surface peptidases	Dipeptidylpeptidase-IV/CD26 Aminopeptidase N/CD13	Enterocytes
		Mastocytes
Tetraspanins	CD63	B-cells
		Dendritic cells
		Enterocytes
		Platelets
		T-cells
Heat shock proteins	CD37, CD53, CD61, CD62 CD9 HSC70 HSP64/90	Mastocytes
		B-cells
		Dendritic cells
		Reticulocytes
		Dendritic cells
		Tumor cells
		Dendritic cells
		Enterocytes

Abbreviation: EMVs, exosomes and microvesicles.

discussion.⁹⁶ Antagomirs represent a new class of chemicals, which have been successfully used to downregulate the endogenous expression of miRNAs.⁹⁷ They represent chemically modified small synthetic RNA oligonucleotides perfectly complementary to the selected miRNA target but mispairing at the cleavage site of argonaute 2 or harboring some modified bases to interfere with argonaute 2 cleavage. Furthermore, antagomirs must be protected from rapid degradation, which is usually accomplished by the introduction of 2' methoxy groups or phosphothioates. The molecular mechanism by which antagomirs block the miRNA function remains to be elucidated, but it may involve irreversible binding to the targeted miRNA.^{97,98} Alternatively, miRNAs can be inactivated, even in vivo, upon administration of so-called “locked” nucleic acids.^{99–101} On the contrary, the endogenous function of a selected miRNA can be recapitulated upon introduction into the target cells of synthetic miRNA mimics consisting of a “guide

Table 4 Some protein components identified in EMVs from various cell types^{303,322,361,363,372–375}

Protein class	Name	Cell type
Cytoskeletal proteins	Actin	Dendritic cells
		Enterocytes
		Mastocytes
	Actin-binding proteins (cofilin)	Dendritic cells
		Dendritic cells
		Enterocytes
Membrane transport/fusion	Annexin I, II, IV, V, VI Annexin VI RAB7, RAPIB, RAB-GDI	Dendritic cells
		Mastocytes
		Dendritic cells
Signal transduction	Gi2alpha, I4–3-3 CBL, LCK	Dendritic cells
		T-cells
Metabolic enzymes	Enolase-I Thioredoxine peroxidase	Enterocytes
		Dendritic cells

Abbreviation: EMVs, exosomes and microvesicles.

strand,” which is identical to that of the selected miRNA and a “passenger strand,” which is chemically linked to a “carrier” molecule, such as cholesterol, for facilitating cellular uptake. Both strands have to be chemically modified to increase their stability to a considerable extent.

In summary, there is increasing evidence for the potential use of EMVs-associated miRNA signatures in body fluids, particularly peripheral blood, as biomarkers for the prediction of metabolic diseases.¹⁰² In addition, the technologies for the up- and downregulation of miRNA activity introduced for elucidation of its (patho)physiology will open new avenues for the miRNA/EMV-based therapy of metabolic diseases.

GPI-anchored protein components

GPI-anchored proteins are widely distributed among all eukaryotic organisms.^{103–106} They harbor lipidic tail structures that tether them to the outer phospholipid leaflet of plasma membranes and EMVs, respectively, as well as relatively large hydrophilic proteinaceous domains that protrude into extracellular/vesicular spaces (Figure 4). The evolutionary purpose of membrane anchorage via GPI linkage for surface protein expression remains unclear so far. GPI-anchored enzymes, receptors, binding proteins, and structural proteins are known to be involved in a number of different physiological processes and functions, such as catalysis, signal transduction, cell adhesion, and complement regulation (Table 6).^{107–123} GPI-anchored proteins also have a great role in embryogenesis, since abrogation in their biosynthesis results in embryonic lethality. Recently, it was shown that interaction of GPI-anchored EphrinA with its receptor is crucial for closure of the neural tube.¹²²

Table 5 Comparison of composition and biogenesis between microvesicles and exosomes^{376–381}

	Exosomes	Microvesicles
Typical membrane proteins	CD63, TNFR1, GPI-anchored proteins	Integrins, GceI, CD73, metalloproteinases
Typical luminal proteins	Cytokines, annexins	Caspases, annexins, cytokines, growth factors
Typical lipids	Cholesterol, ceramides, lysobisphosphatidic acid	Cholesterol
miRNA	Growth, metabolism	Metabolism
mRNA	Growth factors	Growth factors
SNAREs, Rabs, budding proteins	Preliminary evidence	Unknown
Site of generation	Multivesicular bodies (MVB)	Plasma membrane
Mechanism of generation	Membrane budding and fusion	Membrane budding and fusion
Mode of release	Constitutive, regulated	Regulated
Mechanism of release	Exocytosis of MVB	Plasma membrane budding
Mechanism of sorting	Ceramide-dependent	Unknown
Intracellular storage	Yes	No

First indications that proteins might be attached to plasma membranes by lipidic anchors were reported in 1963 with the finding that bacterial phospholipase can release alkaline phosphatase from cells.¹²⁴ The existence of inositol-containing phospholipid protein anchors was suggested in 1976¹²⁵ and was finally accepted in 1985, when detailed compositional data about Torpedo electric organ and human acetylcholinesterase,^{126,127} rat brain and thymocyte Thy-1,¹²⁸ and *Trypanosoma brucei* variant surface glycoprotein^{129,130} became available. Today, hundreds of GPI-anchored proteins are known, and it is estimated that approximately 0.5% of all proteins in lower and higher eukaryotes are modified in this manner.¹³¹

All GPI anchors share a common glycolipidic core structure.^{105,106} Phosphatidylinositol is glycosidically linked through carbon-6 of the myoinositol ring to the reducing end of a nonacetylated glucosamine moiety. Three mannosyl residues, linked to α 1-4, α 1-6, and α 1-2, respectively, are attached to this glucosamine. The terminal α 1-2 linked mannose is linked to phosphoethanolamine via a phosphodiester linkage. The GPI anchor becomes attached to

the carboxyl terminus of the protein by an amide linkage to the amino group of phosphoethanolamine. This common core structure can be further modified in a way that depends on both the organism and the cell type in which it is synthesized.¹⁰⁴

The whole process of GPI biosynthesis is carried out in the endoplasmic reticulum,¹⁰⁶ and nearly 20 enzymes are involved in this pathway. Corresponding genes have been cloned from mammals, yeast, and protozoa.^{132,133} The initial step, the attachment of N-acetylglucosamine to phosphatidylinositol, depends on the product of the X-chromosomal gene, phosphatidylinositolglycan class A (PIG-A) in humans.¹³⁴ A deficiency in PIG-A results in a rare human disease called paroxysmal nocturnal hemoglobinuria (PNH).^{134–137} Patients with PNH have abnormal cells of various hematopoietic lineages that are defective in the biosynthesis of GPI-anchored proteins. These include the complement-regulatory proteins, CD55 and CD59, whose absence results in enhanced complement-mediated lysis.^{138,139} Since deficiency of GPI biosynthesis is embryonically lethal,^{119–121} all PNH patients reported to date

Table 6 Some representatives for GPI-anchored proteins^{382–387}

Enzymes	Receptors	Others
Alkaline phosphatase	Folate-binding protein	Thy-I
Acetylcholinesterase	CD14	CD24
5'-nucleotidase (CD73)	CD16	CD55 (DAF)
Alkaline phosphodiesterase I	CD48	CD58
cAMP-specific phosphodiesterase (GceI)	Plasmodium transferrin receptor	Tamm-Horsfall glycoprotein
Renal dipeptidase (MDP)	Urokinase receptor	Carcinoembryonic antigen
Aminopeptidase P	CNTF receptor	Prions (PrPC, PrPSc)
NAD ⁺ glycohydrolase	Nogo-66 receptor	NCAM-120fs
Carboxypeptidase M		Ly6 family (CD59, Ly6A)
Carbonic anhydrase IV		
ADP-ribosyltransferase		

Abbreviations: GPI, glycosylphosphatidylinositol.

apparently have acquired a somatic mutation in PIG-A.¹⁴⁰ The exact mechanism how one or a few of the large number of pluripotent hematopoietic stem cells that bear a mutation in PIG-A achieve dominance in the bone marrow and the peripheral blood is not known so far.¹⁴¹ Possibly, PIG-A deficient cells have lower susceptibility to TNF- α and IFN- γ , and this resistance might contribute to their clonal dominance.

Once the biosynthesis of the GPI anchor is completed, it is transferred to a specific site upstream of the carboxy-terminal end of the protein in the endoplasmic reticulum lumen by the action of a transamidase complex, which simultaneously cleaves off the remaining carboxy-terminal peptide.^{132,133} A specific signal for GPI anchor attachment has been identified at the carboxy terminus of the protein moiety.^{103,106} After attachment of the prefabricated GPI anchor, the GPI-modified proteins are then transferred from the endoplasmic reticulum to the Golgi complex where they are subjected to further modifications at their GPI moiety. Finally, they are transferred to the plasma membrane via the trans-Golgi network and secretory vesicles as mature GPI-anchored proteins. In mammalian polarized cells, the GPI-anchored proteins seem to be targeted predominantly to the basolateral plasma membrane domain.¹⁴²

Although the polypeptide moieties attached to the GPI anchors do not apparently share common features, the presence of the GPI anchor itself appears to confer some important characteristics on them. In particular, localization to plasma membrane microdomains or lipid rafts – which are highly enriched in (glyco)sphingolipids, cholesterol, saturated fatty acids, and certain proteins, serving as platforms for a variety of cellular functions, such as vesicular trafficking^{143,144} and signal transduction (Table 7) – appears to play critical roles in the transduction of signals across the plasma membrane, the translocation of GPI-anchored proteins from plasma membrane lipid rafts onto cytoplasmic lipid droplets (LDs),^{145–147} and the release of GPI-anchored proteins into EMVs.^{148,149} The clustering of GPI-anchored proteins in lipid rafts^{150–161} may be required for the initial budding, subsequent shedding, and final release of EMVs enriched in GPI-anchored proteins.

Release and transfer of EMV-associated GPI-anchored proteins

In addition to liberation by enzymatic, ie, lipolytic or proteolytic cleavage, GPI-anchored proteins can be released

Table 7 Some selected signaling pathways mediated by lipid rafts and major components involved^{388–394}

Signaling pathway	Major component
Humoral immune response	B-cell receptor
Growth, differentiation	EGF receptor
Nitric oxide	Endothelial NOS
Immune response	Fc ϵ R1 receptor
Insulin, glucose metabolism	Insulin receptor
Antidiabetic therapy	Glimepiride-induced phospholipase C
Insulin-mimetic signaling	Phosphoinositolglycans
Cell adhesion	Integrins
Cellular immune response	T-cell receptor

from the outer leaflet of the plasma membrane with their GPI anchors remaining intact. This release can occur via embedding the GPI-anchored proteins into small aggregates together with some membrane phospholipids.¹⁶² Alternatively and more frequently, the release is provoked through EMVs. Little is known about the signals that target proteins, such as GPI- and other lipid-anchored, eg, acylated proteins to the site(s) of EMV release. Candidate signals represent amino-terminal acylation or myristoylation tags, internal phosphatidylinositol-(3,4,5)-bisphosphate-binding domains, carboxy-terminal prenylation, and palmitoylation tags as well as type-I integral plasma membrane outer leaflet targeting domains.^{80,83,163,164} With regard to GPI-anchored proteins, their segregation into the site(s) of EMV release may rely on the intrinsic capability of GPI anchors to spontaneously insert, accumulate, and aggregate in lipid rafts of the outer plasma membrane leaflet.¹⁶⁵

The phenomenon of the release of polypeptides from donor cells and subsequent intercellular transfer to and uptake by acceptor cells of a GPI-anchored protein were reported even before the actual discoveries of GPI anchors and the presence of GPI-anchored proteins in EMVs. While investigating phospholipid exchange between cells and artificial vesicles and liposomes, ACE and some other erythrocyte proteins were observed to be transferred from the erythrocytes to the vesicles and liposomes in reversible fashion.¹⁶⁶ The rate, direction, and extent of those apparent intermembrane transfers were found to depend on the relative phospholipid composition and fluidity of both the donor and the acceptor membranes.¹⁶⁷ In addition, the spontaneous insertion of exogenously added purified human decay accelerating factor (DAF) into the surface of sheep erythrocytes was reported,¹⁶⁸ which resulted in freely mobile and fully active DAF, as demonstrated by its inhibition of convertase complexes and mediation of resistance to complement-mediated lysis.¹⁶⁹

Since then, a number of other GPI-anchored proteins were successfully incorporated into a variety of different cell types in vitro under retention of the same characteristics and functions as their endogenously expressed counterparts.^{162,167–180} For instance, CD59 was transferred from seminal plasma to erythrocytes and other cells,^{179,181,182} as well as from erythrocytes to endothelial cells in mice made transgenic for this GPI-anchored protein.^{169,183} Storage of erythrocytes resulted in the loss of both CD55 and CD59 from the erythrocyte membrane¹⁸³ and generation of erythrocyte EMVs that are enriched in GPI-anchored proteins, including CD55 and CD59.¹⁸⁴ Interestingly, CD59 incorporated into U937 monocytic cells and allowed to equilibrate for 2 hours at 37°C showed redistribution into lipid rafts and signaling via intracellular Ca^{2+} fluxes.¹⁸⁵ Therefore, exogenously added GPI-anchored proteins appear to become functional within the target cell membrane once they have acquired a distribution similar to that of their endogenous counterparts during a slow process that can take even more than 24 hours.^{186,187} Incubation of rat Thy-1 antigen with murine lymphocytes showed that the rat protein was transferred to murine cells and incorporated into their plasma membranes, where the exogenous protein migrated with the same lateral mobility as endogenous murine Thy-1 protein.¹⁸⁸ Similarly, incorporation of *T. brucei* variant surface glycoprotein (VSG) into baby hamster kidney cells showed that the inserted VSG exhibited lateral mobility equivalent to that of endogenous VSG in *T. brucei*.¹⁸⁹

When erythrocytes from PNH patients who were deficient in GPI-anchored proteins were incubated with high-density lipoprotein (HDL) preparations or erythrocyte EMVs from normal blood donors, significant transfer of CD55 and CD59 to the cell surface occurred. Pretreatment of the EMVs and HDL with bacterial phosphatidylinositol-specific phospholipase C abrogated protein transfer to CD55/59-deficient cells, indicating that the elevated cell-associated CD55/59 levels were related to the insertion of an intact GPI anchor into the outer leaflet of the plasma membrane by the GPI fatty acyl chains rather than to simple adhesion.¹⁸⁴ An elegant experiment successfully demonstrated the ability of GPI-anchored proteins to transfer between cells in vivo.¹⁷² PNH patients of blood group A1 were given transfusions of compatible washed group 0 blood. Patients' group A1 cells were distinguished from the transfused group 0 cells by staining with a *Dolichos biflorus* lectin that specifically binds to group A1 erythrocytes. Significant transfer of GPI-anchored proteins from donor cells to patients' erythrocytes could be demonstrated as

early as one day following transfusion and persisted for several days.^{190,191}

Transfer between membranes can occur without actual membrane fusion.¹⁶² The GPI-anchored proteins are apparently transferred through EMVs released from the donor cells.¹⁹² Moreover, GPI-anchored proteins were reported not to transfer spontaneously from erythrocytes to liposomes or between plasma membranes in vivo.¹⁹² The involvement of a catalyst was supported by the observation that CD4 engineered to become biosynthetically coupled to a GPI anchor is efficiently transferred between plasma membranes in one type of cell,¹⁹³ while another cell line expressing CD4-GPI fusion protein failed to release it in any form.¹⁹⁴ With regard to CD59, HDL may act as its carrier and manage to transfer CD59 to erythrocytes.¹⁹⁵ The exact mechanism(s) underlying the EMV-mediated transfer of GPI-anchored proteins, which may involve receptor-receptor interactions, direct fusion or endocytosis of the EMVs by the acceptor cell, subsequent fusion with endosomes (Figure 7), and identification of the hypothetical catalyst are currently under intense investigation,^{169,173,185–188,196} but in any case, they rely on the intactness of both GPI lipid and protein moieties.^{168,186} This conclusion was drawn from the observation that transgenic mice expressing the GPI anchor-less (released instead of cell surface-expressed) version of the prion protein (PrP) were infected with the pathogenic scrapie form of PrP, but they never developed manifest prion disease.¹⁹⁷ Experiments with cells expressing anchorless PrP were also resistant to scrapie infection.¹⁹⁸ Apparently, lack of the GPI anchor on PrP would prevent its transfer and could explain why cells expressing GPI anchor-less PrP were unable to sustain a scrapie infection over time.

(Patho)physiological roles of EMV-associated GPI-anchored proteins

The transport of GPI-anchored proteins and miRNAs across the plasma membrane and their intracellular translocation onto cytoplasmic LDs (GPI-anchored proteins, only) or release into the circulation through EMVs (GPI-anchored proteins and miRNAs) as well as the subsequent transfer from the EMVs of the donor cell to the plasma membrane of an acceptor cell were independently discovered and rediscovered in several distinct research areas during the past decades and termed shedding, release, incorporation, painting, uptake, jumping, transfer, and translocation.^{62,159,162,192,199–201} In these studies, the differential release and transfer of EMVs originating from a multitude of cell types were implicated with the pathogenesis of a broad variety of diseases.

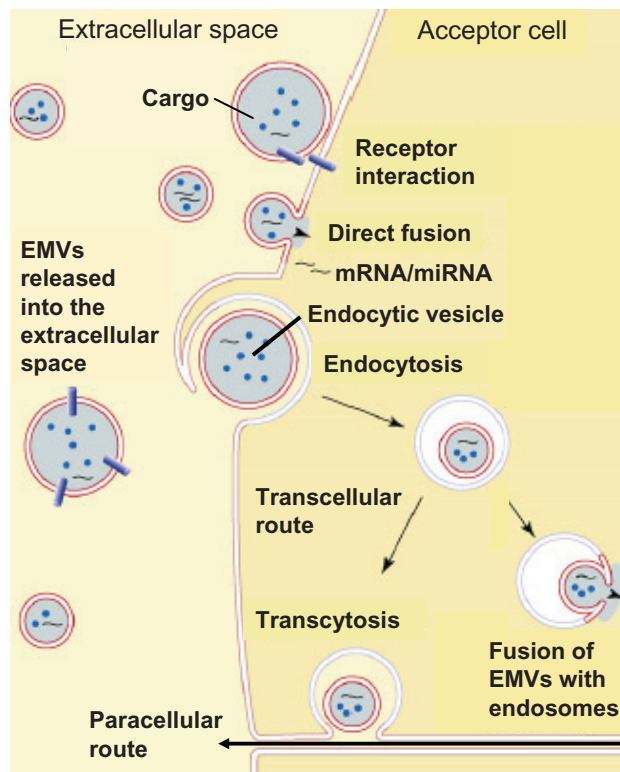


Figure 7 Current working hypotheses for the different modes of interaction of EMVs released from donor cells into the extracellular space with target acceptor cells.

Notes: May involve receptor interaction, direct fusion or endocytosis. Upon fusion of the endocytic vesicle membrane with the EMV membrane, the EMV contents become delivered to the cytoplasm of the acceptor cell. Alternatively, in polarized epithelial or endothelial cells the endocytic vesicle upon budding from the apical plasma membrane may undergo transcytosis and fuse with the basolateral plasma membrane. This transcellular pathway, as well as the paracellular route across cell-connecting tight junctions, mediate the transport of EMVs released from tissue cells into body fluids. © 2008, Elsevier. Adapted with permission from Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2008;19:43–51.⁴⁷

Abbreviation: EMVs, exosomes and microvesicles.

One prominent source of EMVs is tumor cells that use the release and transfer of certain EMV components to evade destruction by immune cells.^{52,201} Retroviruses exploit transfer via EMVs for spreading to other cells.^{200–203} However, these extensively studied mechanisms are actually examples of a misuse of EMVs and some of their components. The real reason that this process developed in the course of evolution has yet to be elucidated. One reported physiological function is the transfer of GPI-anchored proteins and gangliosides by prostasomes from prostate epithelium to spermatozoa.¹⁶² Since spermatozoa do not synthesize proteins, the transfer may represent an important target cell-oriented mechanism by which spermatozoa can obtain new proteins and alter their antigenicity or acquire resistance to immune attack and other surface properties. Another rather probable function of the transfer is the modulation of the composition of lipid rafts and their function, eg, in signal transduction. Together

with the GPI-anchored proteins, gangliosides are located in lipid rafts. Even though they arise from different biochemical pathways, they both have lipid anchors that tether them to the outer leaflet of plasma membranes and enable their release from the donor cell and transfer to the acceptor cell in a regulated fashion.⁵⁸ Since GPI-anchored proteins and gangliosides are both localized in lipid rafts, they could affect each other. Exogenous administration of gangliosides affected the distribution of GPI-anchored proteins within lipid rafts.^{203–206} It is tempting to speculate that modification of lipid rafts by removal or addition of specific gangliosides might create favorable conditions for the release of GPI-anchored proteins.

In rodent adipose tissues, the release of (GPI-anchored) proteins and mi/mRNAs from donor adipocytes and subsequent transfer to acceptor adipocytes via EMVs is known to exert multiple (patho)physiological consequences.^{207–211} Recently, the fusion of EMVs derived from large white rat adipocytes and harboring the GPI-anchored proteins, Gce1 and CD73, the mRNAs coding for the fat-specific (LD-associated) protein, FSP27, and the lipid-synthesizing enzyme, glycerol-3-phosphate acyltransferase (GPAT3), as well as the microRNAs, miR-16 and miR-222, with distinct small adipocytes was demonstrated to stimulate lipid synthesis and storage, in parallel with inhibition of lipid degradation through lipolysis.^{207–211} Importantly, the release of those EMVs from human and rat donor adipocytes was upregulated by physiological signals, such as palmitate and H₂O₂, as well as pharmacological signals, such as the antidiabetic sulfonylurea drug, glimepiride.^{207–212} Furthermore, there is experimental evidence that this induced release is epigenetically controlled via demethylation of histone H3 at lysine 4 and dual methylation of histone H3 at lysine 9 within the promoter of the tetraspanin protein, CD9 (Müller G et al, unpublished data). Ectopic expression of CD9 and silencing of endogenous CD9 expression in human adipocytes triggered significant upregulation and downregulation, respectively, of the release of EMVs harboring CD73 and CD9 proteins, FSP27/CideC mRNA and miR-222. This was accompanied by downregulation and upregulation of lipid degradation and oxidation, respectively. In agreement, lipid synthesis and the expression of the LD-associated proteins FSP27/CideC and perilipin A were decreased and increased upon up- and downregulation of CD9 expression, respectively. Lipolysis, LD-associated Gce1 protein and its cAMP-specific phosphodiesterase activity, the oxygen consumption rate, the extracellular acidification rate, and the expression of the mitochondrial proteins, uncoupling

protein 1 (UCP1) and F_1 -ATPase, became elevated and diminished in response to up- and downregulated CD9 expression, respectively (Müller G et al, unpublished data). Together these findings suggest that upregulation of the release of EMVs harboring specific GPI-anchored proteins, mRNAs and miRNAs, is under the control of differential histone methylation of the CD9 promoter, which leads to fostered CD9 expression and ultimately triggers lipolysis and oxidation of the fatty acid products in (newly generated and partially uncoupled) mitochondria. This raises the possibility that upregulation of EMV release and transfer within human adipose tissue depots may represent a novel target for the therapy of obesity.^{213,214}

Unfortunately, the phenomenon of transfer of GPI-anchored proteins via EMVs is difficult to discover and track for the following reasons: (1) different GPI-anchored proteins undergo different mechanisms of release and its regulation; (2) transfer efficacy is often rather limited, at least if total GPI-anchored proteins are analyzed; (3) transfer is directed to only a limited number of GPI-anchored proteins and/or specific cell types; (4) from a technological point of view, it is difficult to detect the transfer of GPI-anchored proteins in whole organisms. In conclusion, the physiological significance and function of release and transfer of EMVs are not well understood at present. It will be interesting to learn whether and, if so, how they support the integration of individual cells within and between tissues.

(Patho)physiological roles of EMV-associated mi/mRNAs

Considering the importance of miRNAs as an inevitable cornerstone of the human genetic system, the engagement of EMVs for the transfer of genetic material could be an efficient method within the human body to exchange biological information. EMVs containing miRNAs would enable intercellular and inter-organ communication in the body.²¹⁵ Consequently, EMVs could shuttle mRNAs and miRNAs directly from donor to acceptor cells, thereby considerably increasing the probability that the transferred genetic information would affect the function of the acceptor cells upon their successful expression and silencing functions, respectively. The release of mi/mRNA-harboring EMVs may enable the cell-to-cell communication irrespective of the distance between the cells within an organ or tissue or between cells of different, more or less remote organs and tissues. The identification of EMVs in blood and various body fluids hints at the possibility that this exchange of genetic information between organs or tissues may involve EMVs.²¹⁵

Moreover, the membrane of EMVs harbors donor cell-specific factors (eg, GPI-anchored proteins) that enable the EMVs to target specific acceptor cells for transfer of their mi/mRNA contents with exquisite specificity and efficacy. Those EMV-associated mi/mRNAs may reflect disease-specific causal mechanisms. Experimental evidence is emerging that the prediction, diagnosis and prognosis of certain diseases may be facilitated by measurement of the levels of specific mi/mRNAs in EMVs isolated from certain body fluids.

Initial credit for this attractive possibility was gained during cancer research.^{216–226} Certain tumors were recently demonstrated to shed EMVs, which are rich in signaling molecules and genetic material that together constitute a specific and readily identifiable signature.⁴⁷ Moreover, miRNAs entrapped in EMVs were detected in the serum of cancer patients by quantitative real-time polymerase chain reaction in tumor-specific fashion.^{58,221} The miRNA profiles from circulating tumor EMVs were found unique and distinct compared to those from normal controls.⁸⁸ Similarly, specific miRNA profiles have been reported for EMVs isolated from patients with lung cancer, glioblastoma, and hepatocellular carcinoma.⁵⁷ In lung adenocarcinoma, the total tissue miRNA signatures differ considerably between cancer patients and normal probands, and the profiles of the EMV-associated miRNAs resemble very closely those isolated from the tumors.⁵⁷ Tumor-derived EMVs are known to transfer mRNAs to monocytes within the tumor microenvironment to stimulate these cells for the production of cytokines, resulting in enhanced tumor growth and dampening of the immune response.²²⁰ EMV-associated miRNAs released from hepatocellular carcinoma cells were shown to induce the downregulation of the transforming growth factor- β activated kinase-1 (TAK1) signaling pathway in hepatocarcinogenesis, and thereby may cause the local spreading, intrahepatic metastases, and multifocal growth in hepatocellular carcinoma.²¹⁸ Tumor-released EMVs were found to be a prerequisite for the promotion of tumor metastasis in the course of proinflammatory cytokine-driven proliferation of myeloid-derived suppressor cells via the MyD88 pathway.²²³ EMVs released from Epstein–Barr virus-infected B95–8 LCL cells and isolated from monocyte-derived dendritic cells were found to induce gene silencing in the acceptor cells.²²² Macrophages have been reported to increase the invasiveness of breast cancer cells by EMV-mediating transfer of oncogenic miRNAs from the macrophages to potential cancer cells.²²⁵ mRNAs from endothelial-derived EMVs have been found to exert proangiogenic effects.²²⁶

An additional link between EMVs and miRNAs has recently become apparent in immunology and virology research. EMVs released from human and murine mast cell lines were found to harbor over 1200 mRNA and about 121 miRNA species.²¹⁵ EMVs released from donor dendritic cells were reported to dock to and fuse with target dendritic cells and to transfer their contents into the target cells under accompanying specific silencing of the miRNA-targeted genes.²²⁷ miRNA-harboring EMVs released from stromal cells have been shown to support quiescence of B-cells. The apparent transfer of miRNAs from bone marrow stroma to B-cells could be involved in the dormancy of bone marrow metastases.²²⁴ The presence of viral miRNAs in EMVs is evidence that they may function as vesicular carriers for spreading of the disease or initiation of the infection process.^{228,229} Thus, there is increasing evidence that EMV-associated miRNAs function in the acceptor cells following their transfer. Based on the unique and specific signatures of the transferred EMV-associated miRNAs, their use as biomarkers in screening tests for the prediction and prognosis of cancer and other diseases has been proposed.

EMVs as biomarkers

General considerations

The following characteristics argue for the potential of EMVs as a promising source for new biomarkers: (1) EMVs are identifiable and isolatable on the basis of typical intrinsic and well-defined properties, such as phosphatidylserine content, size, sedimentation behavior; (2) EMVs are specific with regard to the expression of cell-lineage markers as well as the overall molecular composition and patterns of their luminal and surface contents; (3) EMV signatures critically depend on the stimulation and micro-environment of the donor cells; (4) EMVs are initial and rapid “responders” since they are released early during stimulatory or micro-environmental changes and in the pathogenic cascade of a disease; (5) EMVs are noninvasive since they are detectable in many body fluids; (6) EMVs are “translatable” since their release is not limited to one cell type or one species; and (7) EMVs act as vectors since they transport and protect biological messages, ie, mRNAs, microRNAs, proteins, and phospholipids, which are normally confined to cells/tissues.

It is well established that for each type of biomarker, its predictive value increases with the number of members of this biomarker type combined as well as with the relative proximity of the time points of biomarker measurement, ie, disease prediction and onset of the disease, ie, diagnosis

(Figure 2). On the basis of the specific structural and functional features of EMVs (see “EMVs as biomarkers: Type II diabetes” and “Obesity”), it seems reasonable (albeit still speculative) to assume that EMVs enable prediction with higher probability (compared to genotypic biomarkers) and at earlier time points (compared to phenotypic biomarkers) along the complete pathogenetic pathway, including the initiation, further development, diagnosis, further progression, and outcome of the disease.

Origins and sources

During the past two decades, the existence of EMVs has been demonstrated in blood and a range of other body fluids, such as urine, saliva, mucus, and breast milk. Consequently, there is an increasing interest in their potential use as tissue-borne and easily accessible diagnostic biomarkers for many diseases, including cancer, cardiovascular diseases, and metabolic diseases.^{116,117,230–242} As a prerequisite for the use of EMVs as multi-component biomarkers reflecting different dysfunctional or disease stages of those relevant cells/tissues from which they originate, it is important to understand the critical parameters and molecular mechanisms involved in their passage from the donor cells into the corresponding body fluid (Figure 7).

Current estimates of the concentration of EMVs in peripheral blood of healthy probands are 5–50 µg/mL. Although the term “microparticles” is neither specific nor fully descriptive, it has been used for the past 30 years and unfortunately remains standard in the current literature on EMVs of the blood.^{84,242–248} Using flow cytometry, it was found that the majority of the peripheral blood EMVs from normal individuals are actually derived from blood cells, particularly platelets (Figures 8 and 9).^{249,250} For instance, about 80% of the EMVs analyzed in human plasma samples obtained during a clinical study were determined to be of platelet origin,²⁵¹ and the number of nonplatelet-derived (eg, tissue-derived) EMVs were well correlated to the clinical data and disease history of the patients. Initially, platelet-derived EMVs were considered to have pathophysiological importance since they expose a multitude of procoagulant anionic phospholipids, such as phosphatidylserine, in a similar fashion as activated platelets and consequently have been the most extensively studied.^{84,244,247,252} In the 1940s, it was demonstrated that clotting of platelet-deprived plasma was delayed after high-speed centrifugation.²⁵³ This observation suggested that procoagulant subcellular structures are present in plasma and are removed by sedimentation. Surprisingly, the release of membrane fragments from activated platelets,

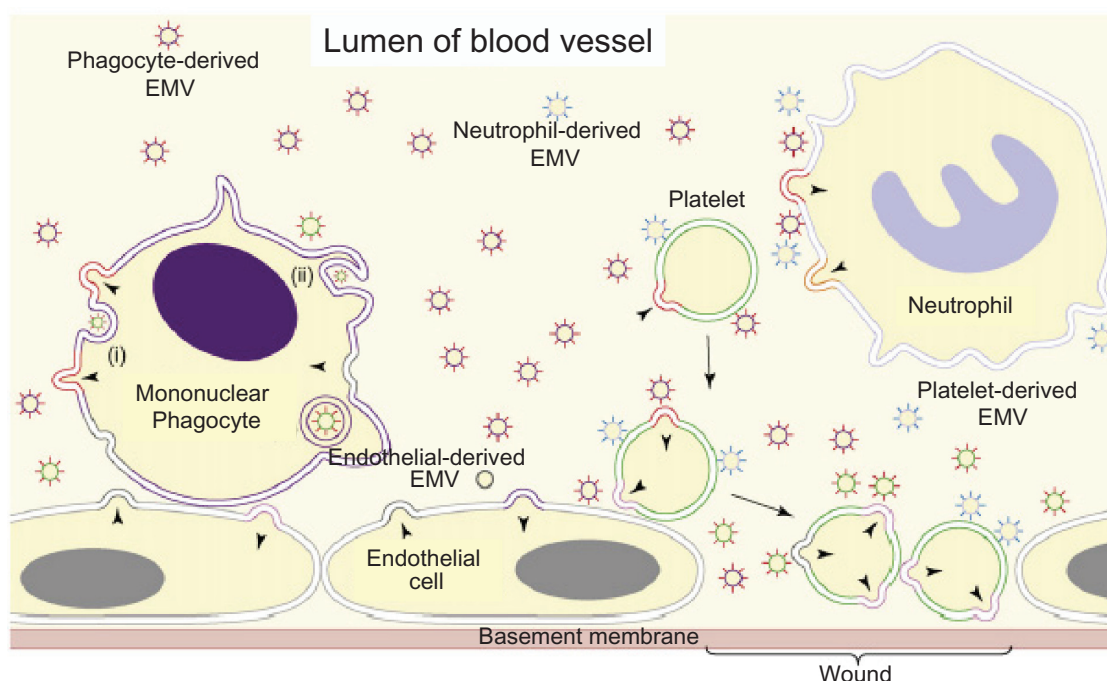


Figure 8 Schematic depiction of the origin of EMVs in the blood. © 2008, Elsevier. Adapted with permission from Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2008;19:43–51.⁴⁷

Abbreviation: EMVs, exosomes and microvesicles.

thereafter called “platelet dust,” was not demonstrated until 1967.²⁵⁴

The second-largest population of blood EMVs is derived from the mononuclear phagocyte cell lineage (Figures 8 and 9). In contrast, only a small percentage of the peripheral blood EMVs originates from T-cells and

neutrophils. A small subpopulation of blood EMVs seems to be released from endothelial cells according to the expression of corresponding cell surface antigens,²⁵¹ in contrast to the massive EMV production observed with human endothelial cells in vitro.^{255–257} B-cells do not seem to contribute to EMVs to any significant degree.

In addition to blood, urine is one of the most useful sources for EMVs, which are assumed to be released by tubular epithelial cells. It is of tremendous advantage that their collection is simple and noninvasive by nature. Urinary EMVs, the majority of which are thought to represent exosomes, have recently been the subject of intense proteomic analyses.^{258–260} Interestingly, aquaporin-2 has recently been identified in urinary EMVs, but not in EMVs from other sources.²⁶¹ Thus, aquaporin-2 positive EMVs may be useful as biomarkers for renal dysfunction and structural injury as they typically develop during diabetic nephropathy.

The transport pathway of platelet-, blood cell-, and endothelial cell-derived as well as tubular epithelial cell-derived EMVs into the circulation and urine, respectively, appears to be straightforward. In contrast, the mechanism of how EMVs released from tissue cells reach the blood is intriguing since these EMVs have to transit vascular endothelial cells during their passage from the interstitial space into the circulation. The transport of EMVs across

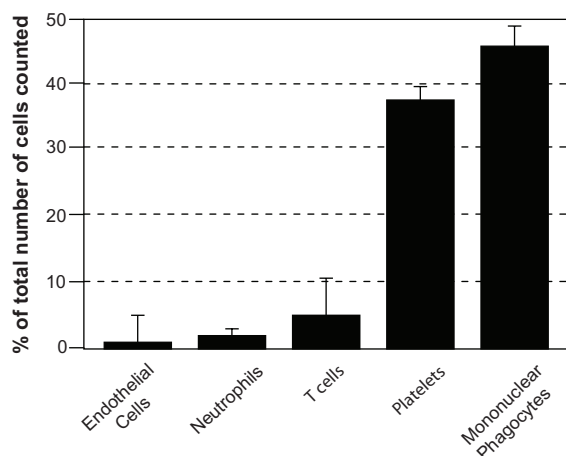


Figure 9 Analysis of the origin of peripheral blood EMVs.

Notes: EMVs from the peripheral blood of healthy donors were analyzed by flow cytometry on the basis of staining for CD3, CD202b, CD66b, CD79a and CD41a to delineate their release from T-cells, endothelial cells, neutrophils, B-cells and platelets, respectively, or for CD14, CD206, CCR3, CCR2/5 to reveal their release from mononuclear phagocytes.^{406–412}

Abbreviation: EMVs, exosomes and microvesicles.

endothelial cells may occur via (1) the transcellular route involving transport vesicles, which are generated by endocytosis of the plasma membrane and subsequently fuse with the opposite plasma membrane; or (2) the paracellular route involving the transient opening of tight junctions in course of direct interaction with certain protein components (Figure 7). Recently, the operation of a third pathway has become conceivable based on the observation that small leucine-rich proteoglycans, such as biglycan and decorin, as secreted components of the extracellular matrix critically affect the communication between neighboring tissue cells in the course of their continuous formation and degradation.^{262–264} Hence, biglycan and decorin seem to regulate the active and passive transport of nutrient and hormones^{265,266} and possibly also of EMVs. Interestingly, biglycan was found to be elevated in obese atherosclerotic mice,²⁶⁷ in rats with metabolic syndrome, and in type II diabetes patients^{268,269} compared to controls. Its prominent expression in stromal vascular cells of visceral adipose tissue suggests that the expression level of biglycan and other components of the extracellular matrix may modulate the efficacy of EMV transfer from donor to acceptor cells within a tissue depot (eg, from large to small adipocytes) as well as of EMV transport from donor cells into body fluids (eg, from adipocytes into plasma). These pathways and the underlying molecular mechanisms must be delineated for a better understanding of the correlation between the levels of EMVs in body fluids and the pathogenesis of metabolic diseases.

Type II diabetes

Preclinical studies aimed at finding evidence for the potential usefulness of EMVs as type II diabetes biomarkers demonstrated that: (1) db/db mice display significantly higher levels of circulating total and platelet-derived (but not tissue-derived) EMVs compared to db/+ mice; (2) male db/db mice treated for 2 weeks with antidiabetic compound and exhibiting significantly elevated body weight gain, diminished blood glucose levels, increased plasma adiponectin levels, and decreased plasma insulin levels have significantly reduced amounts of total and tissue-derived (but not platelet-derived) EMVs; (3) streptozotocin-induced mice exhibit significantly increased levels of circulating total, platelet-derived and tissue-derived EMVs prior to the onset of fully established diabetes; and (4) circulating EMVs increase in levels and change their signature between healthy volunteers and patients suffering from type II diabetes^{270–272} or metabolic syndrome.^{272–278}

The development of vasculopathies and endothelial dysfunction during type II diabetes is known to involve multifactorial processes, including the pathological activation of vascular cells. The release of EMVs by activated cells was reported predominantly for diseases associated with thrombotic risk, but few data are available for type II diabetes. Platelet-derived EMVs of both CD42⁺ and CD41a⁺ phenotypes as well as monocyte-derived EMVs (CD14⁺) were found elevated in patients with type II diabetes.²⁷⁹ In particular, the latter EMVs were highly increased in patients suffering from diabetic nephropathy and may be indicators of vascular complications.^{280,281} Another study demonstrated sustained, elevated amounts of circulating platelet-derived procoagulant EMVs (CD42⁺) after acute myocardial infarction in diabetic patients, which were higher than in nondiabetic patients with myocardial infarction.²⁸² Tissue factor-positive EMVs associated with T_H cell-derived (CD4⁺), granulocyte-derived (CD66e⁺) or platelet-derived (CD61⁺) antigen were significantly increased in early type II diabetes when compared with healthy control subjects.²⁸³ Importantly, EMV numbers did not correlate with *in vivo* coagulation markers, but correlations were found between various tissue factor-positive EMV subpopulations and several components of the metabolic syndrome, such as body mass index, fasting plasma glucose, insulin, TNF- α , and serum HDL cholesterol. This hints at a diabetogenic role that is distinct from the procoagulant role of the detected tissue factor antigen.

In addition, significant differences have been demonstrated in the number and the procoagulant activity of circulating EMVs between type I and II diabetic patients.^{280,284,285} When compared with control subjects, type I diabetic patients presented significantly higher numbers of platelet-derived EMVs (CD41⁺), endothelial cell-derived EMVs (CD51⁺), and total phosphatidylserine-positive EMVs as well as elevated levels of EMV-associated procoagulant activity. In contrast, in type II diabetic patients, only the number in phosphatidylserine-positive EMVs was significantly higher without concomitant increase of their procoagulant activity.²⁸⁶ Importantly, in type I diabetic patients, only procoagulant activity associated with phosphatidylserine-positive EMV was correlated with the level of glycated hemoglobin, suggesting that procoagulant activity is associated with impaired glucose tolerance and homeostasis.²⁸⁷

Previous reports have indicated that high levels of plasma dipeptidyl peptidase-IV (DPP-IV), also known as CD26, are positively correlated with type II diabetes. DPP-IV degrades the active form of the incretin, glucagon-like peptide-1,

which is released from intestinal L-cells after meal intake and enhances insulin secretion in a glucose-dependent manner (Table 3). DPP-IV inhibition causes blood glucose decrease in animal models of diabetes and type II diabetic patients. The DPP-IV inhibitors sitagliptin and vildagliptin are currently widely used as an adjunct to diet and exercise to improve glycemic control in type II diabetic patients.^{288,289} DPP-IV is a membrane-associated peptidase highly expressed in the brush border and microvillar fractions of the kidney cortex. Recent findings showed that in urine, EMV-associated DPP-IV represents the major portion of urinary DPP-IV activity.^{290,291} The excretion of this urinary EMV-associated DPP-IV was significantly higher in type II diabetic patients compared to control probands.²⁹¹ The urinary levels of EMV-associated DPP-IV were found to be positively correlated with the urinary albumin/creatinine ratio in type II diabetic patients.^{292,293} These results suggest that the urinary level of EMV-associated DPP-IV is associated with and may be used for the prediction of the development and outcome of diabetic nephropathy.

The potential of EMV-associated miRNAs as biomarkers for type II diabetes can be indirectly inferred from the known multiple roles of miRNAs in the regulation of lipid and glucose metabolism. These were revealed by a multitude of *in vitro* and *in vivo* (mice) studies, which used the delivery of miRNA precursors or antagomirs⁹⁷ into cells to overexpress or silence the miRNAs of interest and to evaluate their application to therapy.^{294,295} For instance, the blockade of miR-122 expression by systemic administration of an miR-122 antagomir oligonucleotide caused considerable lowering of plasma cholesterol levels as well as hepatic fatty acid and cholesterol synthesis rates in normal mice, leading to diminished levels of triglycerides and hepatic steatosis in diet-induced obese mice.²⁹⁶ Consequently, the mice appeared to be in good health after 4 weeks of therapy with the antagomirs. Furthermore, the expression of miR-103/107 was found upregulated in obese insulin-resistant *ob/ob* mice.²⁹⁷ The specific and efficient silencing of miR-103/107 in liver and fat by delivery of cholesterol-conjugated anti-miR antagomir significantly improved glucose homeostasis and insulin sensitivity in *ob/ob* as well as diet-induced obese, but not chow-fed wild-type mice compared to scrambled or mismatched antagomirs. Interestingly, as a consequence of the downregulation of miR-103/107, the amount of caveolin-1, a positive regulator of insulin receptor signaling, becomes elevated in parallel with improved insulin signaling, reduced adipocyte size, and upregulated insulin-stimulated glucose transport.²⁹⁷ These findings argue for the potential use of

specific miRNAs as biomarkers for the development of insulin resistance and type II diabetes, in particular, if the miRNAs are incorporated together with other typical EMV components into circulating EMVs.

In summary, the currently available experimental evidence strongly argues for the usefulness of EMVs, particularly those released into the plasma and urine, as specific biomarkers for the pathogenesis of type II diabetes. Moreover, there is an urgent need to detect those EMVs that originate from disease-relevant tissues, are released into accessible body fluids and have already increased in number in the prediabetic state.^{298–300} Putative candidates for relevant EMV donor cells and tissues are pancreatic islets, particularly β -cells, liver, skeletal muscle, adipose tissue, smooth muscle cells, vascular endothelial cells, and tissue and plasma macrophages. The future challenge will be to identify the EMV signatures that are specific to those cells and tissues causally involved in the pathogenesis of type II diabetes and/or reflecting the temporal and individual disease states.

Obesity

The increased prevalence of obesity in present day society and the current view of adipose tissue as one of the most critical regulators of energy homeostasis and metabolism have warranted a sustained interest in studying the mechanisms controlling its formation. In general, obesity can be regarded as the consequence of a long-lasting imbalance between energy intake and energy expenditure, finally leading to the storage of the surplus energy as triacylglycerol in the adipocytes, which thereby undergo both hypertrophy and hyperplasia. Clearly, both adipocyte hypertrophy and hyperplasia is now generally accepted to be involved in the dramatic increase in adipose tissue mass in obese as well as type II diabetic patients.³⁰¹ The underlying molecular mechanisms and defects have not yet been fully elucidated. Interestingly, they may encompass the paracrine exchange of information about the lipogenic vs lipolytic state between large and small adipocytes within adipose tissue depots via adipokines and/or EMVs.^{302,303}

Alterations in the number and size of adipocytes are typically accompanied by changes in the expression patterns for subsets of miRNAs. For instance, during adipocyte differentiation, the levels of miR-130b and miR-210 were found to be decreased in both lean and obese probands. Levels of miR-222 were decreased in lean probands but increased in obese subjects.^{304,305} Levels of miR-103, miR-107, miR-143, and miR-185 were upregulated in the lean state but downregulated in the obese state.^{304,305} Interestingly, from the

miRNAs analyzed so far, only miR-34a has been found to be positively correlated with the rate of adipocyte differentiation and development of the BMI.³⁰⁵ The expression of the majority of these miRNAs is known to be controlled by certain adipokines, such as TNF α that downregulates miR-103 and miR-143 and upregulates miR-221 and miR-222.³⁰⁴

It was recently found that EMVs released from dysfunctional and hypertrophic adipocytes manage to impair the function of vascular endothelial cells, which may have implications for the development of obesity-linked complications. For instance, EMVs released from primary rodent adipocytes were demonstrated to stimulate angiogenesis *in vivo*.³⁰⁶ Strikingly, as revealed by antibody arrays and gelatin zymographic analyses, these EMVs harbored a multitude of angiogenic and antiangiogenic adipokines, growth factors and enzymes, such as leptin, TNF α , acidic fibroblast growth factor- γ and matrix metalloproteases-2/5. Upon incubation with human umbilical vein endothelial cells, these EMV cargo proteins promoted the induction of cell migration and tube formation, but only in combination after the removal of leptin, TNF α , or fibroblast growth factor- γ by neutralizing antibodies, which caused abrogation of the angiogenic effect to a major degree.³⁰⁶ Moreover, a subset of miRNAs (let-7b, miR-103/143/146b/148/155/221) was found associated with those EMVs.³⁰⁷ Surprisingly, let-7b, miR-143, miR-155, and miR-221 are known to play similar roles in the control of cell proliferation, apoptosis, inflammation and angiogenesis in adipose and vascular tissues.³⁰⁸ Consequently, it may be proposed that these tissues communicate via EMVs that are released from donor adipocytes and targeted to vascular endothelial acceptor cells.³⁰⁸ This hypothesis gained further credit upon the recent finding that EMVs isolated from plasma and peripheral

blood monocytes share the expression of 71 miRNAs,⁸⁷ the majority of which regulate the differentiation of blood cells, immune functions, and metabolic pathways. Furthermore, a few other EMV-associated miRNAs derived from adipocytes, endothelial cells, or monocytes have been detected in the plasma and demonstrated to affect the function and phenotype of the corresponding acceptor cell type.^{215,309}

However, in comparison with the approximately 400 studies that revealed differential miRNA expression profiles during different cardiovascular disease states and the limited number of medically attractive EMV-associated miRNAs left for the prediction and prevention of cardiovascular diseases so far,^{309–313} even fewer EMV-associated miRNA candidates have been reported to date as being differentially expressed in and released from adipose tissue cells in the course of obesity development. In particular, miRNAs have also been recognized as regulators of adipocyte metabolic integration, energy homeostasis, and differentiation (Table 8).^{314–315} For instance, the expression of 50 out of 70 miRNAs was found to significantly differ between human pre- or mature adipocytes from lean and obese individuals.³¹¹ Moreover, EMVs released from cultured 3T3-L1 adipocytes have been reported to harbor about 140 miRNAs.³⁰⁵ The majority of them were adipocyte-specific, abundant in correlation with their expression level in the donor cells, and considerably upregulated during adipocyte differentiation *in vitro*.^{316,317} Interestingly, these apparently adipocyte-derived EMVs also seem to mediate the transport of mRNAs coding for adiponectin, resistin and PPAR γ into cultured macrophages and to induce angiogenesis.^{318–321} In addition, adipocyte-specific mRNAs and miRNAs have also been detected in EMVs isolated from rat serum.^{302,322,323}

Table 8 Functions of dysregulated miRNAs in adipose tissue

miRNA	Function	References
let-7	Adipogenesis, angiogenesis, inflammation	94,395,396
miR-17	Adipogenesis, differentiation, insulin resistance	313,397
miR-21	Adipogenesis, apoptosis, inflammation	398
miR-27b	Adipogenesis, adipocyte dysfunction	399
miR-34a	Adipogenesis	305
miR-92	Adipogenesis	397
miR-125b	Adipogenesis, adipocyte dysfunction, inflammation	305
miR-130a	Adipogenesis	400
miR-132	Inflammation, insulin resistance	401,402
miR-143	Adipogenesis, adipocyte dysfunction, proliferation	300,398
miR-155	Apoptosis, inflammation, oxidative stress	401,403
miR-210	Adipogenesis	305
miR-221	Adipogenesis, adipocyte dysfunction, angiogenesis, inflammation	305,404
miR-222	Adipogenesis, adipocyte dysfunction, angiogenesis, inflammation	305,404,405

Moreover, EMV-associated miRNAs seem to play key roles in the proper functioning of adipocytes and vascular endothelial cells as well as adhesion, apoptosis, oxidative stress, hypoxia, and infiltration of inflammatory cells (Table 8). The similarity in the apparent effects of EMV-associated miRNAs on adipose and vascular tissues raises the possibility of the operation of parallel molecular mechanisms in adipocytes, vascular cells, and macrophages. Apparently, lipid storage and handling varies among adipocytes (triacylglycerols), macrophages, and smooth muscle foam cells (cholesterylesters). However, rather than the species of the accumulated lipids, changes in their amount per se may trigger phenotypic and functional alterations. This view was supported by the observation that the initial expansion of adipose tissue is accompanied by the emergence of M2-polarized adipose tissue macrophages, whereas in the course of further adipose tissue enlargement (as a consequence of both adipocyte hypertrophy and hyperplasia), the mere deposition of lipids of the same species in these macrophages induces their M1 polarization.³²⁴ This conversion of the macrophage phenotype associated with obesity-induced insulin resistance, inflammation, and severe oxidative stress may be further enhanced by lowered levels of adiponectin. This adipokine is known to be preferentially released from small instead of fully lipid-loaded adipocytes^{303,325} and to improve the metabolism of macrophages through its antidiabetic and anti-inflammatory activities.³²⁶ Hence, EMVs derived from adipocytes, vascular cells and macrophages, and harboring miRNA constituents seem to reflect the functional state of the adipose tissue and thereby may be useful for following the development of obesity.

The posttranscriptional cascade governing adipogenesis, ie, the differentiation of adipocytes from precursor cells and their subsequent maturation from small unilocular (ie, containing many small LDs) to large multilocular (ie, containing few large LDs) cells has been extensively studied for many years.³²⁷ Novel findings suggest the involvement of EMVs in the control of maturation of rodent adipocytes within adipose tissue depots *in vitro*.^{146–149,300,302,307,321,328} In particular, the release of EMVs from primary and cultured rat and mouse adipocytes has been demonstrated to undergo considerable upregulation upon challenge with physiological stimuli, such as excess of fatty acids (palmitate) and reactive oxygen species (H₂O₂), as well as pharmacological agents, such as the antidiabetic drug, glimepiride.^{206–210} These adipocyte-derived EMVs are thought to regulate lipid metabolism between large and small adipocytes within the same adipose tissue depot

during the transfer of some of their constituent components (Figure 10). Among them are the GPI-anchored cAMP-specific phosphodiesterase, Gce1, and 5'-nucleotidase, CD73 (Table 6), which in concerted fashion catalyze (c) AMP degradation,^{145,146,328} and the mRNAs coding for GPAT3 and FSP27, which drive lipid synthesis and LD biogenesis, respectively,^{329–334} as well as the miRNAs, miR-16, and miR-222, which have been implicated in the coordination of lipid metabolic pathways.^{292,300,322} The underlying mechanisms encompass (1) the release of those EMVs from preferentially large donor adipocytes; (2) the interaction, fusion or endocytosis of those EMVs (Figure 7) with or by, respectively, preferentially small acceptor adipocytes; (3) the translocation of Gce1 and CD73 from the (lipid rafts of the) EMV membranes to intracellular LDs of the acceptor adipocytes as well as the delivery of the GPAT3- and FSP27-encoding mRNAs and miR-16/222 from the EMV lumen to the cytoplasm of the acceptor adipocytes; (4) the degradation of (c)AMP at the LD surface zone by Gce1 and CD73 (Table 6); and, finally, (5) the upregulation of fatty acid esterification at the LDs and downregulation of fatty acid release from the LDs by concerted action of GPAT3 and FSP27 enzymic and structural activities, respectively, by decreasing of the cAMP levels and by miR-16/222 expression in the acceptor adipocytes.^{145–149,207–209,302} These findings strongly argue that EMVs transfer lipogenic and antilipolytic information from large to small adipocytes to shift the burden of lipid loading within adipose tissue depots during certain (patho)physiological states (Figure 10).³²⁸ Among them are excessive fatty acid intake, antidiabetic pharmacotherapy, and exposure to cellular oxidative stress, which are well known to accompany or foster the obese state. The measurement of these adipocyte-derived EMVs in the adipose tissue depots or, more practicably, in the plasma if they became adequately released into this compartment, could provide helpful information for the prediction of obesity long before the body mass index and the waist-to-hip ratio increase considerably. In fact, EMV levels were reported to be increased in the serum of slightly overweight women compared to normal age-matched controls and suggested to account for the significantly increased risk for obesity-linked (eg, thrombotic) complications during later and advanced obesity in this population.^{335,336}

Epigenetic control

To date obesity has been studied predominantly from a thermoenergetic point of view, taking into consideration the imbalance between energy intake and energy expenditure,

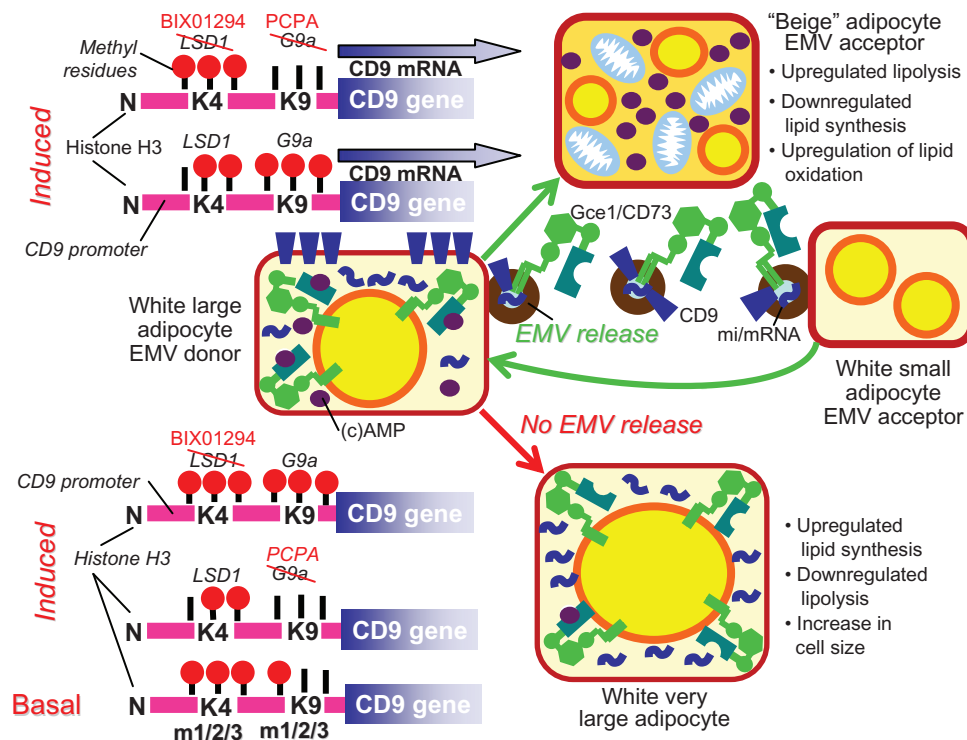


Figure 10 Hypothetical model for the regulation of lipid storage and lipid oxidation in adipose tissue depots consisting of large and small white adipocytes by the release of EMVs harboring miRNAs^{207–212} under the epigenetic control of CD9 expression and their paracrine action (see text for details).

Abbreviation: EMVs, exosomes and microvesicles.

which is determined by complex interrelationships between gene mutations/polymorphisms and lifestyle. The putative involvement of epigenetic mechanisms – ie, of inheritable alterations in gene expression that are manifest in the absence of changes in the DNA nucleotide sequence itself in the development of obesity – has only recently begun to attract attention.^{337,338} The underlying molecular mechanisms include (but are not restricted to) covalent post-translational modifications of the histone amino-terminal tails,^{339–341} in which lysine methylation either enhances or silences the transcriptional state of a given gene, depending on the modified residue. In particular, methylation of lysine 4 of histone H3 (H3K4) usually correlates with gene activation, whereas methylation of lysine 9 of histone H3 (H3K9) is typically associated with transcriptional silencing.^{341–346}

With regard to obesity, an adverse environment during in utero or lactation periods is generally assumed to affect its future development, suggesting that the mother's nutrition or perinatal lifestyle could alter the developmental programming of the fetus and pups.³³⁷ In contrast, the role of adverse environment (eg, nutrition, physical activity, cellular stress, pharmacotherapy) during postnatal growth and adulthood for the modification of the epigenetic pattern remains a matter of debate. Thus, changes in histone methylation patterns

could be the result of the interplay of various dietary and environmental factors and provide a source of interindividual differences with respect to the efficacy of adipogenesis and adipocyte differentiation^{345–349} and ultimately explain the differential susceptibility of genetically similar individuals to develop obesity.^{337,350}

However, it is disappointing that despite the assignment of some miRNAs to critical functions in the epigenetic control of adipogenesis and obesity^{307,322,337,338} and the identification of other miRNAs in EMVs released from adipocytes and circulating in various body fluids,^{302,307,322} experimental evidence for the involvement of EMVs as putative carriers of epigenetic information between somatic cells in para- or endocrine fashion has not been reported so far. However, recent findings of the regulation of cell size and lipid metabolism between large and small rat adipocytes by EMVs harboring specific GPI-anchored proteins, mRNAs, and miRNAs^{300,302} raised the possibility that the transfer of EMVs in response to certain environmental stimuli is under epigenetic control (Figure 10). A recent study demonstrated that in the course of long-term incubation with inhibitors of either H3K9 methylation or H3K4 demethylation, the palmitate-, glimepiride-, and H₂O₂-induced release of those EMVs from large primary rat and differentiated

human adipocytes is significantly reduced.²¹² These results emphasized the putative epigenetic control of adipogenesis with emphasis on the late maturation stage of lipid storage and increased cell size.

The putative functional consequences of the apparent epigenetic regulation of the induced (by fatty acids, oxidative stress, glimepiride) EMV release for large and small adipocytes within an adipose tissue depot can be summarized in the following working model (see also Figure 10): large white donor adipocytes release EMVs harboring the GPI-anchored proteins, CD73 and Gce1, GPAT3 and FSP27 mRNAs, and miR-16 and miR-222. The loss of these EMV components from the large donor adipocytes leads to the upregulation of lipolysis (as a consequence of increased levels of cAMP at the LD surface zone) and downregulation of lipid synthesis (as a consequence of decreased levels of GPAT3 and FSP27 proteins), which trigger the degradation of LDs and eventually the induction of the brown adipose tissue-like phenotype.³⁵¹ The release of these EMVs from the large donor adipocytes is blocked by modulation of their histone H3 methylation state (eg, in the course of G9a or LSD1 inhibition). The retention of these EMV components in the large donor adipocytes supports the downregulation of lipolysis (as a consequence of decreased levels of cAMP at the LD surface zone) and upregulation of lipid synthesis (as a consequence of increased levels of GPAT3 and FSP27), which lead to LD biogenesis and elevation of the cell size. The EMVs fuse preferentially with the plasma membranes of small white (pre)adipocytes. Thereby, the EMVs will deliver the GPAT3/FSP27 mRNAs and miR-16/222 miRNAs into the cytoplasm and translocate the GPI-anchored proteins, CD73/Gce1, onto the LD surface of the acceptor adipocytes. Consequently, GPAT3 and FSP27 protein expression becomes upregulated in parallel with degradation of (c)AMP at the LD surface zone, resulting in the direct stimulation of lipid synthesis and inhibition of lipolysis (Figure 10). The consequent promotion of LD biogenesis drives the increase in cell size of the (initially small) adipocytes and contributes to the stabilization of their white adipose tissue-like phenotype.^{352,353} Upon blockade of the release of the EMVs from large white adipocytes (as provoked experimentally by BIX01294 or tPCPA) or of their transfer to and fusion with the plasma membranes of small white adipocytes (as provoked experimentally by [c]AMP-Sepharose^{208,209} or di-annexin³⁵⁴), the lack of upregulation of FSP27 and GPAT3 protein expression in concert with elevated levels of cAMP at the LD surface zone fosters lipolysis and impairs lipid synthesis. These regulatory events are

linked to the stimulation of mitochondrial biogenesis and blockade of LD biogenesis, respectively, which ultimately lead to the induction of the brown adipose tissue-like or “beige” phenotype (Figure 10). The validity of this working model has to be examined *in vitro* and *in vivo* using human adipocytes and rodent animal models that enable monitoring of adipocyte hypertrophy in response to supraphysiological concentrations of palmitate and reactive oxygen species or pharmacological concentrations of glimepiride.

Based on present and previous findings, it is already conceivable that the release of EMVs from large donor adipocytes or/and their fusion with small acceptor adipocytes within adipose tissue depots can be used as a biomarker tool for the prediction of the epigenetically controlled lipogenic/adipogenic state of this adipose tissue depot, and, in the case of EMV release from the depot into the circulation, of the whole organism. At present, only a limited number of effector molecules and tool compounds are known to modulate EMV release or fusion,^{207–209,355} among them the antidiabetic sulfonylurea drug, glimepiride. The determination of adipocyte-derived EMVs in the blood of type II diabetic patients treated with glimepiride³⁵⁶ could enable the prediction of their risk for developing obesity.

Measurement

General considerations

For the purpose of disease prediction, it is sufficient to find correlations between distinct disease states and subsets of EMVs of specific and unique composition, ie, EMV signatures. Importantly, there is no need for the elucidation of the overall molecular composition of the EMVs (ie, the identity of each constituent) and of their cellular/tissue origin. Of course, this information may turn out to be very valuable for a better understanding of the disease mechanism if the EMVs are causally associated. However, the currently available technologies for the analysis of EMVs rely on their (1) biochemical composition as predominantly revealed by (shotgun) transcriptomics, proteomics and lipidomics and the constituting basic methods of one- and two-dimensional electrophoretic separations, (LC-)MS/MS, nanoLC, Q-TOF, MALDI-TOF/TOF, and state-of-the-art data mining in combination with gene expression studies performed with the candidate donor cells; (2) size, mass and morphology; (3) cellular origin; and (4) flow cytometry.^{318–321,357–363} These technologies manage the biased elucidation of the exact nature of selected constituents, but only in a limited number. It is of crucial

importance to considerably increase the discriminative power of future EMV analytics by the generation of multi-dimensional patterns reflecting the overall constituent signatures of a given EMV species as is typical for type I biomarkers (see “Biomarkers: Definitions”). This demand may be met with the introduction of so-called “nanoparticle tracking” and “chemical noses,” which are already in use for the analysis of complex macromolecules, small vesicles, cell surfaces, and microorganisms for research purposes.^{364–369}

Biosensors

The principal mode of operation of a biosensor for the generation of EMV signatures based on “chemical noses” is as follows (Figure 11A): EMVs isolated from the body fluids of healthy control probands and patients (ie, suffering from type II diabetes) will compete with a selected reporter enzyme, such as glucose oxidase for interaction with nanoparticles. These consist of a gold core with covalently attached tentacle-like aliphatic, positively charged and aromatic substituents

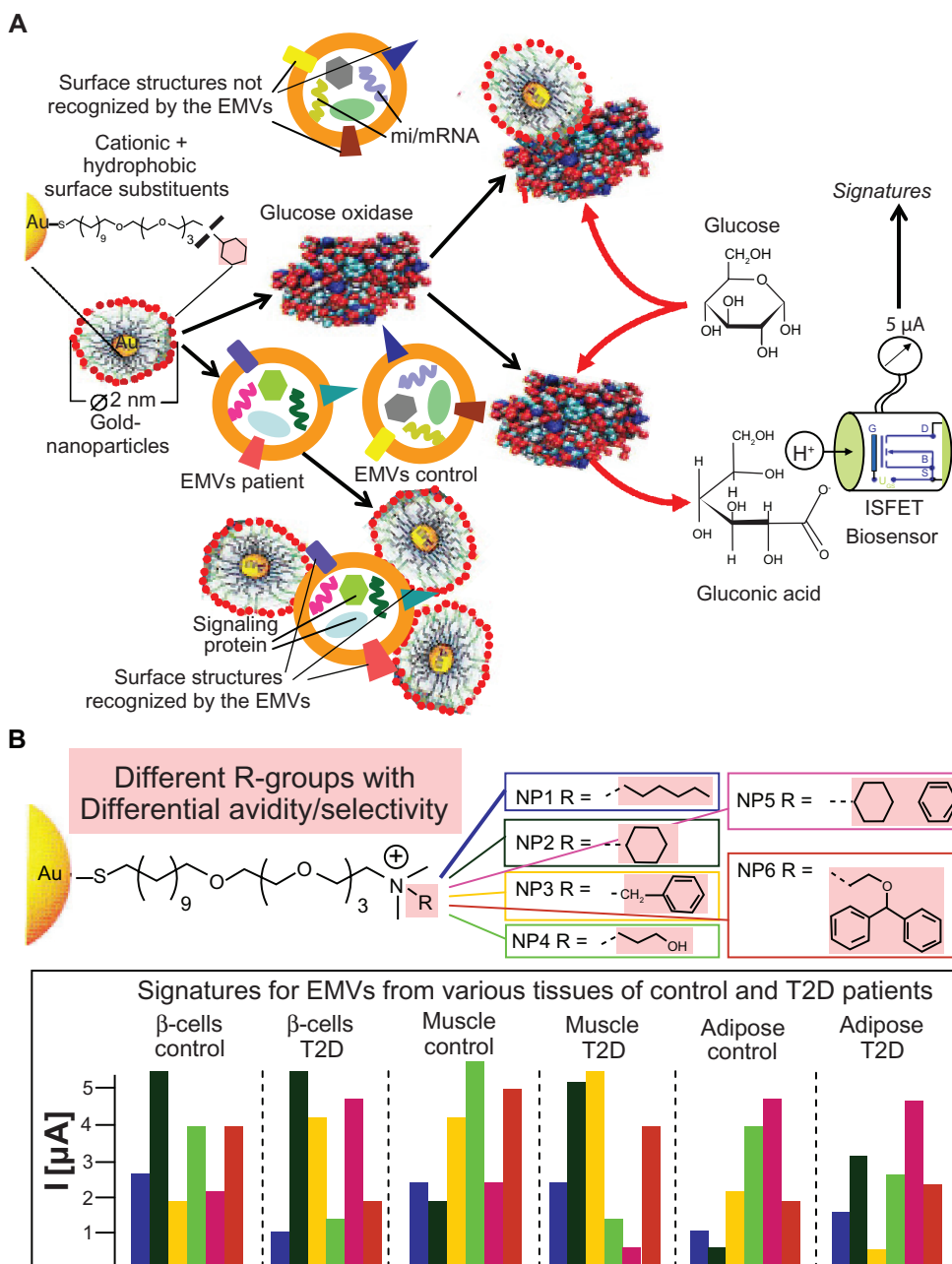


Figure 11 Schematic depictions of the operation of a nanoparticle-based biosensor for the identification of EMV signatures. **(A)** Detection principle. The sizes of the nanoparticles versus EMVs are not drawn at scale. **(B)** Generation of signatures specific for EMVs from different tissues from normal and type II diabetic patients (see text for details).

Abbreviations: EMVs, exosomes and microvesicles; ISFET, ion-sensitive field effect transistor; T2D, type II diabetes.

at their surface, and are capable of interacting with the proteinaceous and lipidic surface structures of EMVs. Moreover, it seems likely that nanoparticles equipped with similar but uncharged substituents can cross the phospholipid bilayer of the EMVs and finally interact with the protein and mi/mRNAs contents in the lumen of the EMVs. The high-avidity interactions of the differentially substituted nanoparticles with the EMV constituents are apparently based on multiple hydrogen, electrostatic, hydrophobic, and van der Waals bonds.

In the case of nanoparticles interacting with glucose oxidase that has higher avidity and specificity than EMVs have, the reporter enzyme becomes inhibited, eg, through steric hindrance of substrate (ie, glucose) access to its binding cleft/catalytic site (Figure 11A). Alternatively, the nanoparticles may prefer interacting with one or the other of the EMVs contained in the sample in differential fashion rather than with each species with the same avidity. Consequently, this nanoparticle species will dissociate from glucose oxidase, relieving it from inhibition. The resulting glucose oxidase activity is quantitatively evaluated by monitoring the generated protons using a biosensor chip based on ion-sensitive, field-effect transistor technology.^{367,368} Upon incorporation into microfluidic cards and lab-on-the-chips, EMV populations can be characterized in a high-throughput format. The measured currents will finally be transformed into EMV-specific signatures (Figure 11B).

A single nanoparticle species will usually not be sufficient for the unequivocal identification of the desired EMV species. More likely, the use of a single or a few nanoparticle species results in more or less similar or even identical signatures due to overlapping recognition patterns and similar surface structures among the various EMV species. However, the discriminative power can be drastically increased by the inclusion of a multitude of nanoparticle species that differ considerably in their surface substituents in hydrophobicity, hydrogen donors/acceptors, and charges. The combination of the different signals provided by separate cycles of biosensor read out will create signatures typical for EMVs from distinct cellular/tissue origin and reflecting the distinct stages of the various disease pathways causing type II diabetes and obesity (Figure 11B).

Conclusion

The physiological function of EMVs is probably intercell signaling through specific interactions with target cells and transferring of biological information encoded in transmembrane, soluble and GPI-anchored proteins,

mRNAs, miRNAs, and phospholipids. Therefore, EMVs may participate in pathogenic processes, such as the development of metabolic diseases. Further insights into the molecular signatures and specificities of distinct EMV species may allow the identification of their cellular origin and contribute to the elucidation of novel drug targets for metabolic diseases. For instance, the removal from plasma of harmful EMVs may be beneficial during the development of type II diabetes and obesity, in which EMVs apparently deliver thrombogenic and inflammatory signals.

In addition, an increasing body of experimental evidence indicates the possibility that EMVs may offer predictive, diagnostic, and prognostic information for metabolic diseases, such as type II diabetes and obesity. The appearance of EMVs in body fluids makes them readily accessible. Their number, cellular origin, composition, and function seem to depend on and correlate with individual disease stages. In fact, EMV levels are positively correlated with type II diabetes and obesity and, in particular, accompany or even induce the development of diabetes- and obesity-linked complications. This finding is based on the demonstration of (1) quantitative differences in circulating EMVs in various animal disease models; (2) changes in circulating EMV levels occurring prior to the onset of atherosclerotic or diabetic symptoms; and (3) differential modulation of the EMV signature in course of pharmacological interference. Analyses of plasma (and, if feasible, tissue) samples from various cohorts of patients at very early to late stages in disease development and recruited for a multitude of clinical studies should be initiated for (1) comparisons between the different pathophysiological states along cross-sectional studies; and (2) follow-up studies for the pathogenesis along longitudinal studies for several years after the recruitment.

On the other hand, and not addressed in this review, EMVs derived from stem cells may reprogram altered functions in target cells, suggesting that they could be exploited in regenerative medicine to repair damaged tissues.³⁷⁰ In addition, EMV-mediated transfer of genetic information could explain the observed plasticity and the functional effects of stem cells without the need for their transdifferentiation into tissue cells.^{371,372}

Many issues remain to be clarified, such as (1) the stimuli and the molecular mechanisms that regulate the assembly of the EMVs and their bioactive load that they transfer from donor to acceptor cells; (2) the stimuli that trigger the release of the EMVs from donor cells; (3) the nature of the surface receptors at the acceptor cells that confer exquisite specificity; (4) the complete diagnostic potential of EMVs for the whole

range of metabolic diseases; (5) the strategies to prevent the formation or to induce the removal from the circulation of potentially harmful EMVs; and (6) the therapeutic exploitation in regenerative medicine of the potency of EMVs to modify the phenotype and function of acceptor cells.

The introduction of detection methods that enable the identification of EMV signatures, in-depth characterization, and specific tracking of EMVs in plasma (and other body fluids) derived from cells and tissues, which are relevant for the pathogenesis of metabolic diseases, should be considerably intensified. It is hoped that recognition of the potential of EMVs will open novel perspectives in the prediction, diagnosis, prognosis, and therapy of metabolic diseases.

Disclosure

The author is an employee of Sanofi Deutschland GmbH and reports no conflicts of interest in this work.

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