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The meaning of the cellular release of glycosylphosphatidylinositol–anchored proteins–Potential use as biomarkers

Abstract

Anchorage of a subset of cell surface proteins in eukaryotic cells from yeast to mammals is mediated by a glycosylphosphatidylinositol (GPI) moiety covalently attached to the carboxy-terminus of the protein moiety. Experimental evidence for the potential of GPI-anchored proteins (GPI-AP) of being released from cells into the extracellular environment has been accumulating, which involves either the loss or retention of the GPI anchor. Release of the GPI-AP may occur in a spontaneous fashion or be regulated by certain endogeneous signals or environmental stimuli. The potential relevance of GPI-AP released from the cell surface into various structural configurations, such as monomers, (homo- or heteromeric) multimers, micelle-like phospholipid complexes, vesicles or lipoprotein-like particles, as innovative biomarkers for disease prediction, diagnosis and stratification is presented. Moreover, the mere description of the release of GPI-AP from cells using modern instrumentation in the absence of any knowledge about the underlying molecular structures and causal mechanisms may be regarded as an example for so-called "hermeneutic phenomenology" within molecular life sciences. An adequate three-step study procedure is discussed. It is based on a known, minimal and carefully considered bias rather than on a strong working hypothesis and should lead to a mere database rather than to a narrative justification of the experimentation as well as to a subsequent minimal interpretation of the database rather than on causal mechanistic explanations and speculative working models as typically presented in scientific publications.

Keywords: alkaline phosphatase, CD59, exosomes, microvesicles, glycosylphosphatidylinositol-anchored proteins, GPI-anchored HDL-binding protein-1, phospholipase C/D, diabetes

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Introduction

The experimental data available unambiguously demonstrate the release of a multitude of glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-AP) from cells of a large panel of eukaryotic organisms which typically relies on the operation of a number of distinct complex molecular mechanisms and the formation of a multitude of distinct structural arrangements, such monomers, (homo- or heteromeric) multimers, extracellular vesicles (EV), lipoprotein-like particles or micelle-like phospholipid complexes, the so-called GPI-AP- and lipid-harboring extracellular complexes (GLEC)(Müller, in press). Less clear so far has remained the functional or (patho)physiological relevance of the release of GPI-AP into the extracellular medium *in vitro*, and interstitial spaces or body fluids *in vivo*. It encompasses (i) the removal of waste (inactivated or unwanted GPI-AP), (ii) the alteration of specific cell surface characteristics (as determined by the GPI-AP), (iii) the intercellular (paracrine or endocrine) transfer of materials or information (manifested in or encoded by the GPI-AP) from donor to acceptor cells and (iv) the biogenesis of extracellular structures and complexes with specific (local or systemic) function (as exerted by the GPI-AP in concert with the other constituents of the structures and complexes). Moreover, at present it seems possible that certain GPI-AP become released to a certain (minor) degree as a consequence of spontaneous or induced (i.e. in response to exogenous environmental cues, such as UV irradiation, or endogenous cell-derived signals, such as differentiation-dependent deformation of the cell shape) alterations of the biophysical properties of the plasma membranes, such as fluidity, surface tension, local curvature, stretching, shearing.

It remains to be studied whether the release of GPI-AP from eukaryotic cells can be used for certain medical or pharmaceutical applications. In principal, the use of GPI-AP as disease biomarkers[1] or for novel biomaterials[2] is conceivable. In particular, vesicular (exosomes, microvesicles), particulate (surfactant-like particles, lipoprotein-like particles, nodal vesicular particles, milk fat globules), multimeric and micelle-like (GLEC) assemblies constituted by GPI-AP equipped with the complete GPI anchor and specific components, such as phospholipids and cholesterol, may offer novel opportunities for the prediction, diagnosis, prognosis and stratification of common (e.g. metabolic) diseases.[3]

GPI-AP as biomarker for common complex diseases

In cases of known (patho) physiological function(s) of a GPI-AP it is tempting to speculate about the consequences of its putative release from the surface of the expressing cells into the corresponding body fluids caused by one of the structure and mechanism described above. Those considerations, in particular in case of relevance for (patho) physiological processes in humans, as is true for the following prominent examples for GPI-AP with known critical roles in the

pathogenesis of human diseases, may justify a closer look at the presence and the levels of released versions of the GPI-AP in relevant body fluids under normal (healthy animals, probands) and pathological conditions (animal models of disease, patients). The observation of GPI-AP release and measurement of levels of soluble GPI-AP should motivate the evaluation of their potential use as biomarkers, in particular in comparison with traditional biomarkers for the delineation of putative advantages of GPI-AP biomarkers. Traditional phenotypic biomarkers for common complex diseases, such as for metabolic diseases, encompass blood glucose, glycated hemoglobin HbA1c, plasma insulin (diabetes) and plasma LDL cholesterol, blood pressure, body weight, BMI (obesity) etc. and - in combination (metabolic syndrome)-reflect the complex interactions between the multitude of susceptibility genes, which for the control of metabolism are currently being estimated to account for at least one third of all human genes, and the multitude of environmental influences, which are determined by the individual life stage and style, and together form a very dense and complex network. For consideration of the environmental impact, so-called phenotypic biomarkers have to be determined, however, preferably and unfortunately at a rather late stage of the pathogenesis, since the earlier the time points of their measurement, the less predictive they are. As a consequence they fail to support disease prediction prior to disease onset or at early phases of the pathogenic development.[4] Moreover, the traditional phenotypic biomarkers typically do not allow stratification of common complex diseases into the multiple subtypes according to distinct underlying pathogenetic mechanisms and the resulting (late) consequences, such as diabetic late complications (nephropathy, retinopathy, neuropathy, microangiopathy, macroangiopathy) in type 2 diabetes (T2D). In conclusion, phenotypic biomarkers often fail to support individualized preventive and therapeutic efforts.[1]

As an alternative, so-called novel molecular biomarkers, predominantly soluble serum proteins, such as cytokines (e.g. TNF-α), adipokines (e.g. leptin), incretins (e.g. GLP-1) and others (e.g. cross-reactive protein) were determined in a number of previous research clinical studies. Interestingly, in combination those novel peptidic biomarkers yielded prediction values approaching but not exceeding those achieved with combinations of traditional phenotypic biomarkers.[1,5] Not surprisingly, the highest prediction probabilities have been reported so far for combinations of traditional phenotypic and novel molecular biomarkers.[6] However, because of the partial overlap of the pathogenic pathways reflected by those biomarkers for a given disease, the predictive values upon their combination did not reach the sum of their individual contributions.[6] It may be argued that the future increase in the number of susceptibility genes identified for common complex diseases in course of genome-wide association studies will lead to considerable improvement of the predictive power of combinations of polymorphic genotypic biomarkers which cover multiple target tissues and pathogenic pathways. In fact, the required considerable improvement of the predictive power is frequently thought to rely on the inclusion of novel (single nucleotide polymorphic) genotypic biomarkers.[7,8] However, the path from genotype to phenotype with the underlying gene-gene and gene-environment interactions, genomic plasticity and epigenetic modifications is of extreme complexity and apparently does not obey simple cause-effect relationships and linear chains of events. Therefore, it remains questionable whether the complete genetic profiling of a given patient for (in theory all) disease susceptibility genes per se will provide information about the operation of a so-called complete causal mechanism (i.e. a chain of successive and interconnected physiological and molecular processes that will be necessary and sufficient to induce and drive the pathogenesis) for a certain complex common disease, such as cardiovascular diseases and T2D, and thereby enable its prediction with the desired probability (i.e. higher than 0.90). These considerations nourish the current hope that the limitations of phenotypic and genotypic profiling could be overcome by novel "complex" biomarkers, such as GPI-AP with complete anchor in specific complex arrangements, which may exceed the potencies of (combinations of) the traditional phenotypic, molecular (peptidic) and genotypic disease biomarkers.

Specific GPI-AP with cleaved or complete anchor

By nature, released GPI-AP with lipolytically or proteolytically cleaved GPI anchor have to be regarded as candidates for molecular (peptidic) biomarkers with their soluble protein moieties corresponding to typical secreted serum polypeptides with regard to their putative (limited) information content. In contrast, GPI-AP released from the cell surface with the complete GPI anchor remaining attached may represent novel "complex" biomarkers of higher information content. This assumption is based on the demonstrated presence of additional biomolecules, such as phospholipids, cholesterol, other (membrane) proteins, in characteristic structural arrangements, such as EV, particles, (heteromeric) multimers and GLEC and the possibility that the composition and configuration as well as the accompanying biophysical characteristics of the complexes undergo changes of differing extent dependent on the (individual) pathogenic development. The resulting variations could reflect differences in the (patho) physiological (e.g. metabolic) state of the releasing cells or donor tissues between probands and/or patients. However, the co-expression of GPI-AP and other components in a complex does not necessarily imply an increase in biomarker "quality" compared to that exerted by the individual entities in "sum". A surplus may arise through the coordinated interaction of the distinct (biophysical) properties and (enzymic, signaling) functions of the individual entities assembled into the complex. If (some of) those hypotheses and assumptions would turn out to be true in the future on the basis of significant correlations in course of longitudinal and cross-sectional research and clinical studies, GPI-AP equipped with the complete GPI anchor and assembled into complexes will be useful for the prediction of the initiation, diagnosis of the onset and prognosis of the progression as well as endpoint of a common complex disease, including the desirable stratification between affected individuals. However, at present severe methodological constraints of the reported studies often do not allow the clear-cut differentiation as to whether the measured serum levels of a given GPI-AP refer to the released protein moiety lacking the GPI anchor (and other entities) and thus to a peptidic biomarker, only, or are indicative for complexes harboring GPI-AP with complete anchor and additional lipidic (and proteinaceous) constituents and thus for a novel "complex" biomarker. In fact, a research or clinical study devoted to the differentiation of monomeric (hydrophilic) GPI-AP *vs*. multimeric (amphipatic) GPI-AP in complex in body fluids of probands and patients has not been presented so far.

CD59

A typical example for the wide distribution in distinct body fluids of a GPI-AP and its occurrence as amphipatic and/or hydrophilic version

displaying and lacking its GPI anchor, respectively, is provided by human CD59 (protectin), a complement regulatory glycoprotein of 18-20 kDa, which is expressed at the surface of red blood cells, leucocytes,[9,10] platelets[11,12] as well as epithelial and endothelial cells of various origin.[13–15] It interferes with the complement-mediated cell lysis by insertion into the forming membrane attack complex C5b-8, thereby competing with C9-binding to phospholipid membrane bilayers and its polymerization.[16,17] In addition, CD59 is detectable at varying concentrations in human body fluids, such as plasma,[18,19] sweat and tears,[18] colostrum and milk,[20] cerebrospinal fluid,[21] amniotic fluid[22] and seminal plasma.[23] A soluble anchor-less and glycated version of CD59 generated by the action of an (unknown) phospholipase C (PLC) was measured in human serum and urine at concentrations of 100ng/ml and 4µg/ml, respectively, applying a newly introduced and thoroughly validated ELISA with optimized precision, accuracy, reproducibility and sensitivity, suitable for clinical use.[24] The use of this assay for the determination of the glycated version of CD59, formed by non-enzymic glycation of lysine41 in course of blood glucose at high concentration, in the serum of normal and diabetic probands demonstrated that the level of soluble glycated CD59 (i) is significantly elevated in diabetic patients compared to healthy controls, (ii) is independently associated with and positively correlated to the level of glycated hemoglobin HbA1c and (iii) enables the identification of patients with diabetes with high sensitivity, specificity and predictive power. On basis of a putative contribution of soluble glycated CD59 to the pathogenesis of diabetic late complications as causative biomarker (assuming functional impairment due to its glycation), the determination of the serum levels of glycated CD59 should be useful for the prediction and management of diabetes. In addition to the recognition of a correlative relationship between serum CD59 and diabetes, the concentrations of unglycated CD59 as measured by Western blotting, dot blotting or ELISA turned out to be rather low in normal human serum or plasma under basal conditions (7.8 ±6.3ng/ml),[25,26] but to be elevated in plasma of patients after myocardial infarction (27.3ng/ml),[26] during development of melanomas[27,28] and in the urine of patients with idiopathic glomerulonephritis.[29] This may be related to the measured reduced levels of GPI-anchored complement inhibitors during early complement activation in human and experimental diabetic retinopathy.[30] Strikingly, these values increased by 5- to 9-fold upon addition of the detergent octylglucoside to the sample diluent.[31] This may be explained with the detergent causing solubilization of vesicular membranes or phospholipid monolayers or dissociation of multimers or micelle-like complexes or unmasking of an antibody epitope of the GPI-AP in course of its liberation from a binding or carrier protein (e.g. albumin). In other words, octylglucoside could lead to disruption of structures which mediate between the GPI anchor and the aqueous environmental milieu. In fact, experimentally prepared radiolabeled GPI-anchored CD59, but not soluble anchor-less urinary CD59 was shown to be incorporated into high density lipoprotein (HDL) particles upon incubation with human serum as analyzed by high resolution gel filtration and anti-apoA-I affinity chromatography.[32] Interestingly, only a very minor portion of CD59 managed to insert into the low density lipoprotein (LDL) fraction. This unambiguous differential behavior of CD59 in the *in vitro* assembly of lipoprotein-like particles (LLP) with insertion into the corresponding outer phospholipid monolayer seems to be mimicked *in vivo* since immunoaffinity purification and immunoblotting analysis revealed that HDL, but not LPL, prepared from normolipidemic human serum harbors CD59, albeit at low amounts.[32]

Thus it is well conceivable that GPI-AP liberated from associated phospholipids, cholesterol or polypeptides (i.e. other GPI-AP, carrier or binding proteins) are evaluated under these optimized conditions with greater sensitivity and accuracy compared to GPI-AP in the native state. In addition, this detergent effect argues for the presence of CD59 with complete GPI anchor in heteromeric, multimeric, particle-like, micelle-like or vesicular assemblies in human plasma and may even be used as indicator for complex formation of GPI-AP in body fluids, in general. In contrast, the ELISA performed in the absence of detergent may preferentially or even exclusively measure the proteolytically or lipolytically cleaved soluble hydrophilic versions of GPI-AP, not masked by complex constituents. This view is compatible with the finding that in seminal plasma the portion of total CD59 measured with the detergent-free ELISA (about 10%) was recovered almost quantitatively from the aqueous phase upon partitioning between a water/Triton X-114 solution[23,33] and thus obviously reflected the soluble anchor-less portion of CD59. In other words, the detergent-free ELISA failed to detect the GPI-anchored and complex-associated CD59.

Alkaline phosphatase (AP)

Instead of using antibody-based assays, such as ELISA, for the detection of GPI-AP in body fluids, such as blood and urine, the measurement of catalytic activity in case of enzymic nature of the GPI-AP protein moiety may provide evidence for the release of a given GPI-AP from cells or tissues into the circulation, irrespective of whether it harbors or lacks the GPI anchor. For instance, feeding of adult rats with high-fat (corn oil) diet for seven hours resulted in a 2- to 3-fold increase in membrane-free intestinal AP activity in serum.[34] This was qualitatively mimicked by subcutaneous injection of bethanecol or cholecystokinin with peak values reached after 7.5 min and 60 min, respectively. Importantly, the serum intestinal AP did display the characteristics typical for a soluble rather than membrane-associated entity.[14] Taken together, the data obtained with rats imply that bethanecol and cholecystokinin trigger elevations of AP activity in intestinal mucosal surface scrapings of the same order of magnitude as associated with particulate materials which appears in the blood immediately thereafter.[14] These data are compatible with the release of intestinal AP from the enterocytes into the intestinal lumen and in parallel (or shortly thereafter) into the circulation by means of particles. In an effort to characterize the pathway responsible for the trafficking of intestinal AP to the serum and to confirm or exclude the possibility of lumenal membrane vesiculation as a critical step herein, the release of AP was investigated in the ileum-derived human colonic carcinoma Caco-2 cell line. A tightly sealed monolayer of differentiated post-confluent cells of this continuous cell line, grown on a semi-permeable membrane, enabled the analysis of the surrounding medium fluids which correspond to either the apical or the basolateral surface compartment.[35] Interestingly, newly synthesized intestinal AP protein and activity were recovered from both the apical (25%) and basolateral (75%) serum-free medium. Moreover, a considerable portion of the released AP remained associated with membranes as manifested by its sedimentation (105.000xg, 1h) and partitioning into the detergent-enriched phase upon TX-114 partitioning. This behavior allowed the exclusion of proteolytic or lipolytic release of AP from the cell surface. Strikingly, the GPI-AP carcinoembryonic antigen (CEA) was recovered from the serum-free medium in parallel and in a hydrophilic and soluble version, exclusively.[36] On the basis of these cell culture studies it was suggested that intestinal AP becomes released from the basolateral surface of enterocytes and subsequently

moves through diffusion into the apical lumen via passage across the tight junctions and then into the serum compartment *via* transport through the lamina propria.[37] Consequently, the next experimental steps addressed the identification of (a) morphological correlate(s) operating along such a pathway. For this, the *in vivo* high-fat fed rat was used as a model on basis of its high rate of secretion of intestinal AP. Luminal washings of the rat intestine yielded membranous lamellar particles with surfactant-like properties, as revealed by lowering of the surface tension in a pulsating bubble assay, similar to surfactant-like particles (SLP), from which the intestinal AP became liberated by treatment with bacterial PI-specific PLC.[38] Immunogold labeling of osmicated and non-osmicated sections demonstrated that intestinal AP is colocalized with these SLP in the cytoplasm of enterocytes.[39] Most importantly and in agreement with the proposed structure of SLP, these particles were found to enclose lipid droplet-like structures and to accompany those along their trafficking throughout the cytoplasm of the enterocytes. Subsequent more detailed analysis of these SLP revealed that the constituting membranes are enriched with phosphatidylcholine which is predominantly equipped with saturated fatty acids.[40] After fat feeding, the SLP and intestinal AP appeared in the intestinal lumen and blood with comparable kinetics, arguing for their colocalization.[41] The buoyant density of these SLP was found to be similar to that of typical rat pulmonary surfactant particles, but to be significantly lower than that of purified plasma membrane vesicles. These SLP expressed the typical digestive enzymes of apical brush border membranes, however, in very different ratios as well as total amounts. Importantly, intestinal AP was reported to be considerably enriched in those SLP *vs*. the apical enterocyte membranes.

These results resemble data for the GPI-AP 5'-nucleotidase (CD73) obtained with various normal and cancer cell lines. Membrane vesicles recovered from the culture medium were found to be enriched with 5'-nucleotidase activity *vs*. other plasma membrane enzymic activities,[42] arguing for the selective release of GPI-AP in course of membrane vesiculation at specific plasma membrane sites or subdomains, candidates of which represent lipid rafts. Interestingly, GPI-AP embedded into either membrane vesicles or SLP after their release can be detected in normal and cholestatic serum as well as in culture medium, supplemented with serum, at high and constant levels despite the presence of large amounts of GPI-specific PLD in serum. Apparently, serum GPI-specific PLD does not play a major role in releasing GPI-AP from relevant native membrane vesicles and SLP *in vivo*. This is in line with the observation that cleavage of the GPI anchor by GPI-specific PLD *in vitro* depends on their presentation in detergent-like milieu or mixed detergent micelles.

GPI-anchored high density lipoprotein binding protein 1 (GPIHBP1)

GPIHBP1 is a GPI-AP that is expressed in capillary endothelial cells as the predominant binding site for lipoprotein lipase (LPL) and engaged in the transcellular transport of LPL, after its synthesis in and secretion from adipose cells, from the surrounding adipocyte and subendothelial interstitial spaces across the capillary endothelial cells into the capillary lumen where LPL remains attached to the surface of the capillary endothelial cells.[43] LPL is avidly bound to heparan sulfate proteoglycans on the surface of capillary endothelial cells,[44,45] from which it can be dissociated into the capillary lumen by the highly sulfated mucopolysaccharide heparin.[46] Here LPL hydrolyzes triglycerides assembled in triglyceride-rich plasma lipoproteins (chylomicrons and very low density lipoproteins). However, since LPL, in contrast to endothelial lipase, which is expressed and secreted by vascular endothelial cells,[47] is synthesized by parenchymal cells, such as myocytes and adipocytes, it has remained a mystery for decades how LPL gains access to the capillary lumen following its synthesis and secretion into the subendothelial interstitial spaces. As the name already suggests, GPIHBP1 is capable of specific binding high-density lipoproteins (HDL), which represents the function used for its initial discovery by expression cloning.[48] GPIHBP1 harbors a GPI anchor for association with the cell surface, from which it can be released by action of bacterial PI-specific PLC. The subsequent development of GPIHBP1 knockout mice and GPIHBP1 overexpressing cell lines strongly suggested that GPIHBP1 (i) is critical for the lipolytic processing of triglyceride-rich lipoproteins,[49] (ii) binds LPL avidly,[50] (iii) interacts indirectly with triglyceride-rich lipoproteins which seems to be mediated by LPL,[51] (iv) is involved in the delivery of lipoproteins to peripheral tissues[52] and (v) is required for the transendothelial transport of LPL.[49,53] Strikingly, in the GPIHBP1 knockout mice LPL was found to be mislocalized to the interstitial spaces surrounding the LPL-producing parenchymal cells and to be absent from the capillary lumen.[54] Direct evidence for a role of GPIHBP1 in the transport of LPL was derived from the measurement of transendothelial transport using confluent monolayers of cultured endothelial cells, which express GPIHBP1. Strikingly, a monoclonal antibody directed against GPIHBP1 was found to be transported from the basolateral to the apical medium compartments.[54] Convincingly, the release of GPIHBP1 from the basolateral surface of the endothelial cells by GPI anchor cleavage with bacterial PI-specific PLC led to significant impairment of the transendothelial transport of the antibody. Since GPIHBP1 heterozygous mice displaying about 50% GPIHBP1 expression level have normal plasma lipoprotein levels, pathological GPIHBP1 deficiency seems to be a recessive syndrome.[55] Half-normal levels of GPIHBP1 apparently manage to mediate the transendothelial transport of LPL to a sufficient degree in both mice and humans.[56–58]

Of great relevance for the (patho)physiological situation in humans was the observation that GPIHBP1 deficiency in homozygous knockout mice with complete mistargeting of LPL after its initial secretion from striated muscle and adipose tissue leading to residence in the interstitial spaces[59,60] was associated with severe hypertriglyceridemia and chylomicronemia.[54] Subsequently, this phenotype was also recognized in humans to be caused by mutations in either GPIHBP1[50,61,62] or LPL[63] which both lead to impairment of their mutual interaction. Interestingly, homozygous GPIHBP1 knockout mice were found to suffer from lipid- and macrophage-rich atherosclerotic lesions in the aortic root and coronary arteries.[64] The manifestation of this phenotype in humans with GPIHBP1 or LPL mutations remains to be demonstrated. Taken these findings together, an updated model for the lipolysis of triglyceride-rich lipoproteins by LPL in concert with GPIHBP1 was presented:[52] GPIHBP1, GPI-anchored at the basolateral surface of capillary endothelial cells, "picks up" with high affinity soluble LPL from the subendothelial interstitial space following its detachment from GPI-anchored heparan sulfate proteoglycans at the myocyte and adipocyte surface. Subsequently, GPIHBP1 actively shuttles LPL from the basolateral to the apical surface of the vascular endothelial cells with exposure towards the capillary lumen. The molecular mechanism underlying this shuttle remains to be clarified, but may depend on the lateral movement of the GPIHBP1-LPL complex along the basolateral and apical plasma membranes via passage across the tight junctions between

neighboring endothelial cells and between the apical and basolateral plasma membrane domains. Alternatively, transendothelial transport of GPIHBP1 in concert with LPL could proceed from the basolateral domain, following its initial expression, across the cytoplasm of the endothelial cells for exposure of the complex at their apical surface. The underlying transcytotic process may involve caveolae, arising from small invaginations, subsequent budding into the cytoplasm, incision and fusion of the plasma membranes to yield vesicles, which are covered by the atypical monotopic membrane and coat protein caveolin-1.[65] Caveolae have been implicated to mediate transcytosis of fluids, small molecules and proteins in endothelial and epithelial cells.[66] Following arrival at the apical surface of the endothelial cells, LPL remains bound to GPIHBP1 initially, but then becomes detached upon interaction with triglyceride-rich particles[45] or in response to fatty acids generated by lipolysis of the triglyceride-rich particles.[43]

In conclusion, the GPI-AP GPIHBP1 plays an important role in the complex pathway of intravascular processing of triglyceride-rich lipoproteins as is also reflected in the consequences of the demonstrated artificial and naturally occurring defects herein for the lipid metabolism and cardiovascular system during human health and disease. This raises the interesting question about the (patho) physiological effects of the putative release of GPIHBP1 from the basolateral or apical surface of the capillary endothelial cells into the subendothelial interstitial space and capillary lumen, respectively, involving one of the molecular mechanisms and structures described above. Albeit the appearance of GPIHBP1 harboring or lacking the complete GPI anchor in the circulation of mice or humans has not been reported so far, preliminary data indicate that this GPI-AP is a minor constituent of GLEC released into the plasma of diet-induced and genetically obese rats and obese and diabetic humans. Thus it may be attractive to study whether the pathogenesis of metabolic diseases, such as T2D and obesity, is correlated to the levels of circulating GPIHBP1 in any configuration, i.e. as soluble, vesicle-associated, lipoprotein-associated, micelle-like or multimeric version.

Total GPI-AP

The release of a subset of GPI-AP or even of a single GPI-AP entity, such as CD59, AP and GPIHBP1, only, rather than of all members of the GPI-AP family may rely on the type of the molecular mechanism engaged. Removal of the GPI anchor by proteolytic or lipolytic cleavage through a specific protease or phospholipase or interaction of the GPI anchor with a specific carrier protein may favor the release of specific GPI-AP entities. In contrast, the insertion of the GPI anchor into the phospholipid bilayer membranes of vesicles or phospholipid monolayers of particles as well as the aggregation of the GPI anchor in hetero- or homomultimers and in micelle-like complexes may support the release of each GPI-AP under the control of the corresponding mechanism, irrespective of the nature of their protein and anchor moieties. The formation of exosomes, microvesicles and particles, such as SLP, lipoprotein-like particles, nodal vesicular particles or milk fat globules, with GPI-AP as major constituents may be compatible with the release of both specific GPI-AP, subsets of GPI-AP and total GPI-AP, depending on the GPI-AP expression profile of the releasing cells and donor tissues. Thus it may be useful to introduce analytical methods for the unbiased determination of the GPI-AP in total rather than for the measurement of single and specific GPI-AP members in body fluids. Thereby total GPI-AP could be evaluated and validated as biomarkers which become released in course of operation of the seemingly "unspecific" mechanisms for the formation of homomultimers and micelle-like complexes.

Expression level of GPI-AP in the circulation

It was recognized that tumor cells release GPI-AP, among them CEA and mesothelin, from their surface into the circulation with higher efficacy compared to their normal counterparts. Which GPI-AP become released in specific or unspecific fashion as well as the underlying molecular mechanisms and structures remain to be elucidated as is true for the configuration (as soluble, monomeric, multimeric, vesicular, particulate,,micelle-like entities) of the tumor-expressed GPI-AP which enter the circulatory system. The question of the release of GPI anchor-harboring *vs*. lacking GPI-AP was tackled on basis of the GPI anchor remnants left at the released GPI-AP moiety. The proteolytically released hydrophilic version of a GPI-AP lacking any anchor building blocks, including the terminal phosphoethanolamine residue (i), can be discriminated from the lipolytically released hydrophilic version lacking the anchor diacylglycerol (through PLC action) or phosphatidate (through PLD action) residue (ii) and the released amphipatic version harboring the complete anchor embedded in vesicular, particulate, homo-/heteromultimeric or micelle-like complexes (iii), since only the latter two (ii, iii) display the (phospho)inositolglycan core at their carboxy-terminus.

he experimental discrimination between (i) and (ii) can be achieved with the aid of the lectin α-toxin from *Clostridium septicum*, a member of the aerolysin-like pore-forming toxins, that interacts with GPI-AP with high specificity and affinity.[67] Initially α-toxin was used for the efficient capturing and identifying of GPI-AP by mass spectrometry.[68] Importantly, the capturing was not impaired upon lipolytic cleavage of the GPI-AP by bacterial PI-specific PLC and not affected by the very divergent (carboxy-terminal) amino acid sequences. This argues for predominant or even exclusive interaction of α-toxin with the glycan core of the GPI anchor and makes involvement of the protein moiety of the GPI-AP rather unlikely. Moreover, the analysis of serum proteins by mass spectrometry-based proteomics is typically accompanied by considerable hurdles since it necessitates the elimination of abundant polypeptide species and tedious steps of protein fractionation. Thus, the use of α-toxin for the isolation and enrichment of GPI-AP could facilitate their detection upon release into serum and concomitantly enable their classification into proteolytically cleaved versions (i) and those versions with lipolytically cleaved (ii) or complete (iii) GPI anchor. Consequently, in an investigation to evaluate the potential of GPI-AP as biomarkers for certain human cancers, α-toxin was used to measure the level of GPI-AP harboring the glycan core in plasma from patients suffering from several types of tumors.[68,69] Interestingly, the plasma of patients with cancers, that are typically associated with increased mRNA and protein expression of components engaged in GPI-AP biosynthesis, such as the GPIT subunits, displayed significantly elevated α-toxin-binding compared to that of plasma from patients with no malignant disease. Thus the high and very low amounts of total GPI-AP in cancer and control plasma, respectively, as revealed by α-toxin signals suggest that GPI-AP are released (presumably from tumor cells) into plasma as glycan core-containing protein moieties by one or the other of the mechanisms discussed above. In fact, the released glycan core-displaying GPI-AP may be useful as biomarkers for the prediction, detection and stratification of certain cancers. In particular, the GPI-AP FERMT3 and FLNA captured by α-toxin could be relevant with regard to the development of breast carcinomas.[69] Strikingly, based on the criterion of α-toxin-binding, these two GPI-AP were detected in more than 90% of the cancer patients, whereas only very few probands

with non-malignant tumors were tested as positive for the presence of FERMT3 and FLNA in serum.

Surprisingly, in cancers with very high expression of GPIT mRNA and protein, such as ovarian cancer, albeit displaying elevated levels of α-toxin-binding in plasma compared to control plasma, the levels of α-toxin-binding were found to be lower than those measured for other cancers.[69] Apparently, in addition to the expression level of the GPIT subunit genes, other factors seem to be directly involved in the release of those GPI-AP with the glycan core still attached, which as a consequence remain detectable by α-toxin. Alternatively, the GPI anchor glycan core of released GPI-AP may be modified in ovarian rather than in other cancers by factors, such as an endogenous protease or GPI-specific phospholipase, in such a way that it fails to be recognized avidly by α-toxin. Interestingly, high levels of α-toxin-binding to membrane-associated amphiphilic GPI-AP prepared from ovarian cancer tumors as well as from the surface of cells from patient ascites were measured.[70] Together these data hint to considerable structural differences between the GPI anchors of GPI-AP from ovarian tissue, ascites and plasma as explanation for the relatively low α-toxin-binding capacity in the plasma from ovarian cancer patients. A problem often associated with the development of assays for the detection of serum biomarkers relies on the formation of complexes between the protein of interest and certain serum components, which causes masking of the epitopes recognized by the detecting (e.g. ELISA) antibody. In fact, it cannot be excluded that GPI-AP assembled in complexes in serum as described above may escape the traditional detection techniques, such as ELISA, at least under native conditions. This potential limitation would be bypassed in course of isolation of native serum GPI-AP on basis of the interaction of α-toxin with their glycan core. Apparently, this interaction is not susceptible towards masking by serum proteins since no reports about endogenous GPI-binding serum proteins have been reported so far (PubMed). At variance, successful interaction of α-toxin with GPI-AP in the context of complexes such as GLEC Müller et al.,[71] and EV (Müller and Tschöp; data not shown) has been demonstrated recently.

GPI-AP associated with EV

Over the past decades experimental and clinical evidence has been increasing that the presence and accumulation of extracellular vesicles (EV) harboring as major protein components monotopic (e.g. caveolins), bitopic and polytopic (e.g. tetraspanins) transmembrane proteins[72] as well as GPI-AP (e.g. CD73, Gce1) in body fluids, such as blood, liquor, saliva, mucus, urine and interstitial fluids (e.g. pleural fluids, ascites) are related to the pathogenesis of a variety of (common complex) diseases. Experimental evidence is accumulating that the levels of EV and of their (protein) components including GPI-AP in plasma may be related to (cardio)vascular[73–90] and metabolic diseases, in particular T2D and obesity,[91–102] endothelial dysfunction and other common complex diseases.[103–107] These findings can be explained most easily by the biological function of EV in the orchestration of signaling and transport processes, immunomodulation, tissue remodeling as well as the interaction of cells during angiogenesis, cell proliferation and apoptosis/survival.[108–120] Thus, the concentration, composition (also with regard to GPI-AP), cellular origin and biological function of circulating EV may be critical factors in (cardio)vascular and metabolic diseases[121,122] With regard to all these parameters, EV are of considerable heterogeneity. Since 70-90% of the circulating EV is derived from senescent or activated platelets, the transcriptome and proteome of the platelet and platelet-derived EV have to be investigated thoroughly in order to facilitate the discrimination between tissue cell-derived and platelet-derived EV. In conclusion, EV with their complex and variable composition that becomes manifested in their overall signature rather than in single or multiple individual parameters seem to reflect those alterations in gene expression and function of the relevant tissues and cells being involved in the pathogenesis of metabolic diseases and thus could be useful for their prediction, diagnosis, prognosis, therapy monitoring and stratification. In this regard, EV with the embedded GPI-AP can be considered as a type of (patho)physiological "mirrors" rather than classical biomarkers since they combine complete sets of cellular proteinaceous and nucleic acid components which are responsible for certain functional and (patho)physiological consequences characteristic for common complex diseases.

EV-associated GPI-AP and liver diseases

Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by excessive lipid accumulation in the liver which strongly correlates with insulin resistance and its phenotypic manifestations, such as obesity, T2D, dyslipidemia, arterial hypertension.[123,124] It is the most common chronic liver disease in Western countries and encompasses a non-progressive probably benign hepatological form of steatosis and a progressive form of non-alcoholic steatohepatitis (NASH), which significantly increases liver-related as well as cardiovascular mortality, the incidence of T2D and the overall mortality.[125] Currently, the diagnosis is entirely based on histology as revealed by liver biopsies. This is an invasive and cost-intensive procedure and not appropriate for the screening of large numbers of patients at risk for NAFLD. Therefore there is a high medical need for non-invasive diagnosis of NAFLD for the target populations of patients with (i) the progressive form of NASH in order to monitor and propose early therapeutic interventions and (ii) (very) early stages of NASH in order to transfer knowledge about diagnostic and therapeutic options to those suffering from advanced stages of the disease. Both NAFLD and NASH are often associated with obesity, T2D and asymptomatic elevations in serum levels of liver transaminases. The progression of these hepatic diseases is accompanied by the development of endoplasmic reticulum stress[126] and inflammation involving chemokines such as MCP-1, cytokines such as TNF-alpha and metalloproteases. In comparison to NAFLD patients, NASH patients in general are older, more obese and more often have high serum liver enzymes and suffer from T2D and the metabolic syndrome. To date the diagnosis and prognosis of NAFLD/NASH are being performed using ultrasound analysis of the liver for the detection of fatty infiltration, which however does not allow assessment of the degree of inflammation and fibrosis. Therefore, detection of lipids in the liver is easily made by ultrasound, but diagnosis of NAFLD or NASH cannot be performed without liver histology. Several biomarker-based approaches have been proposed to enable the non-invasive diagnosis of liver fibrosis. These are based on panels of serum markers, genetic markers of disease progression, plasma lipidomic signatures, newer imaging methods and breath tests. For instance, a patented algorithm (Fibro-Meter, BioLiveScale, Angers, France) was evaluated and demonstrated improved diagnostic performance for the stages F2-F4 compared to the fibrosis score and AST-to-platelet ratio index.[127] This marker panel index relies on relatively simple and readily accessible parameters, such as age, glucose, liver transaminases, ferritin, platelets and body weight, but

still requires further validation. Plasma lipidomic signatures were also used for the characterization of NAFLD/NASH patients. Even though the combination of various serum markers of liver fibrosis and the results from transient elastography measured by the fibroscan technology had suggested to predict the development of NASH and fibrosis, liver biopsy has remained the accepted gold-standard for the differentiation of NASH and NAFLD.[128] Thus there is still an unmet medical need for the discovery of non-invasive and precise diagnostic tools for patients suffering from these liver diseases.[129]

Tissue remodeling which occurs during NAFLD/NASH may induce the formation of EV containing GPI-AP. Those EV are present in the circulation of healthy individuals at relatively low concentrations. Numerous clinical studies have reported increased plasma EV levels associated with (cardio)vascular risk factors, such as hypertension, smoking, obesity and prediabetes as well as frank (cardio)vascular and metabolic diseases, such as T2D. Concerning liver diseases, an increase of the level of plasma EV was reported in patients with hepatitis C and hepatocellular carcinoma. However, it remains unknown whether liver-derived EV displaying certain GPI-AP were detectable in the plasma of NASH patients. It is commonly accepted that NASH pathogenesis represents a progressive process involving steatosis and inflammation. Therefore it is reasonable to assume that these conditions may result in the release of GPI-AP in association with EV from liver cells, Kupffer cells or (recruited) macrophages. So far, there are no clinical data available linking EV with fatty liver diseases. Nevertheless, in apolipoprotein E2 knock-in mice, which represents an experimental model for atherosclerosis and NASH,[130] the presence of EV was observed both in the atherosclerotic lesions of the vasculature and in the liver. In future projects correlations between the levels of EV displaying GPI-AP in the plasma and the specific stages of NAFLD/NASH have to be delineated by measuring liver cell-specific EV in order to provide and validate biomarker candidates in appropriate clinical cohorts and ultimately to elucidate a relationship between the appearance of specific subsets of GPI-AP-harboring EV and the pathogenesis (with regard to stage and severity) of NAFLD/NASH. The objectives should be as follows: (i) Optimization and routine use of cell culture protocols for the release of EV from human hepatoma, stellate, Kupffer and liver endothelial cell models including cultures of human primary hepatocytes, (ii) identification and validation of markers for GPI-AP-harboring EV originating from hepatic, stellate, Kupffer and liver endothelial cells *in vitro* and *in vivo*, including activation status markers, by using several omics- (e.g. flow cytometry, immune blotting, protein/lipid/DNA arrays, nucleic acid probes) and signature-based approaches, including data mining of public literature, patents and databases, (iii) the measurement and comparison of the total concentrations of EV, their compositions and cellular origins in plasma from patients with different stages of NAFLD/NASH, (iv) the identification of subsets of EV expressing GPI-AP with available biomarkers or imaging methods for monitoring the development of fibrosis and simple steatosis in NAFLD *vs*. NASH with fibrosis and (v) selection of liver cell-derived EV in the circulation for their use as candidate biomarkers in clinical studies. From a methodological point of view, patients suffering from chronic liver disease caused by alcohol misuse, viral infection, autoimmune response or hemochromatosis rather than by NAFLD have to be excluded. All patients should be subjected to a liver biopsy, to detailed hepatological and metabolic phenotyping as well as ultrasound and hepatic elastometry. Based on the results of the liver biopsy, the patients will be categorized into those with (i) steatosis alone, (ii) steatohepatitis (NASH), (iii) NASH and no or minimal fibrosis and (iv) NASH and advanced fibrosis/initial cirrhosis. Differences in the number, size, composition and cellular origin of GPI-AP-expressing EV are expected according to the absence/presence/degree of NASH and the extent of fibrosis and steatosis. If needed, patient sub-groups have to be selected in order to improve putative correlations.

EV-associated GPI-AP and immune diseases

There is increasing evidence that for certain acute lung diseases circulating leucocyte-derived EV that express GPI-AP can be used for their prediction as well as prognosis of the outcome.[131,132] For instance, acute respiratory distress syndrome (ARDS) as the most severe clinical manifestation of acute lung injury occurs in systemic inflammatory response syndromes as well as in sepsis. Evidence from clinical and experimental studies is converging that leucocytes play a pivotal role during the acute phase of ARDS. The local and systemic pro-inflammatory responses, which accompany ARDS and sepsis, apparently are coordinated by EV, which display GPI-AP at their surface and are released from leucocytes, platelets and endothelial cells in response to their interaction.[133] Those EV are commonly regarded as sensitive biomarkers for the assessment of the activation or apoptotic state of cells in systemic inflammatory response syndromes and sepsis. In fact, high levels of leucocyte-derived EV have been identified in the plasma of ARDS patients and associated with better outcome in ARDS.

EV-associated GPI-AP and lipid diseases

Recently gained experimental evidence demonstrated that the transfer of proteins and nucleic acids from releasing (i.e. donor) to target (i.e. acceptor) adipocytes via EV[134,135] is associated with (patho)physiological consequences, such as the upregulation of the esterification of fatty acids into triacylglycerol in target adipocytes and the downregulation of the lipolytic fatty acid release from target adipocytes.[136–141] This apparent phenotype switching was triggered by the mere incubation of (preferentially small) acceptor adipocytes with EV released from (preferentially large) donor adipocytes which harbor the GPI-AP, Gce1 and CD73, as well as the mRNAs coding for fat-specific protein 27 (FSP27) and glycerol-3-phosphate acyltransferase (GPAT3) and the miRNAs, miR-16 and miR-222.[142] Importantly, the release of those EV from large primary rat adipocytes as well as differentiated human adipocytes in response to physiological (palmitate, H_2O_2) and pharmacological (anti-diabetic sulfonylurea drug glimepiride) stimuli was shown to be considerably reduced in the presence of 5-aza-2'-deoxycytidine (5-Aza-CdR).[142] This inhibitor of cytosine methylation as well as the specific inhibitor of histone lysine methyltransferases, BIX01294, are known to induce substantial remodeling of heterochromatic domains. The blockade of EV release by 5-Aza-CdR or BIX01294 did not correlate with an alteration in the apoptotic rate, but was accompanied by impairment of the H_2O_2-(but not insulin-) induced stimulation of esterification and inhibition of lipolysis in large (but not small) primary and differentiated human adipocytes.[142]

In contrast, the simultaneous presence of 5-Aza-CdR and BIX01294 had almost no effect on the palmitate-, glimepiride- and H_2O_2-induced release of those EV and the regulation of lipid metabolism. These findings argue for the modulation of the induced release of EV harboring GPI-AP, mRNAs and miRNAs, that are specific for the

control of lipid metabolism, from rat and human adipocytes by DNA and histone methylation in interdependent fashion.[142] Furthermore, it has been proposed that the EV-mediated transfer of lipogenic and anti-lipolytic information between large and small adipocytes in response to certain physiological and pharmacological stimuli may be inherited by epigenetic mechanisms.[142–144] Consequently, it is tempting to speculate that interference with the epigenetic control of the information transfer between adipocytes as well as between adipocytes and other relevant cells (e.g. monocytes, macrophages, pericytes) modulates the complex molecular mechanisms through which environmental (e.g. special nutritional, hormonal, stress) conditions, especially in early life, lead to a biochemical memory effect that influences the susceptibility towards lipid disorders, including metabolic syndrome, T2D and obesity.[144–149]

The predictive power of classical biomarkers for the prediction of common complex diseases, such as metabolic diseases (lipid disorders, T2D, obesity) is currently not sufficient for the individual differentiation of the pathogenesis, clinical outcome and therapeutic options as well as for the individual monitoring of the therapeutic success. Monitoring of multi-parameter patterns may add a novel level of stratifying quality. EV equipped with GPI-AP in addition to a variety of transmembrane proteins, mRNAs, miRNAs and phospholipids are known to be released from various tissues, such as adipose, liver, and cell types, such as immune cells, into multiple body fluids, such as plasma, saliva and urine[150–153] in differential fashion in response to normal and diabetogenic/obesogenic conditions. EV with their complex and variable composition that is manifested in their "overall-signature" rather than in single or multiple parameters seem to reflect those alterations in gene expression and function of relevant cells and tissues being involved in the pathogenesis of common complex diseases and thus could be useful for their prediction, diagnosis and therapy monitoring.

Information or material transfer by EV?

The research area of EV has been gaining enormous credit in attention by the scientific community as well as funding agencies during the past two decades after more than five decades of minor research interest after their initial description[73] and following the first attribution of a physiological function, which was claimed to rely on waste disposal.[154] Currently, EV are frequently and typically regarded as carriers of biological information between cells, tissues and organs in multicellular organisms and this (hypothetical) function is often interpreted as the only or most physiologically relevant role. In fact, it is tempting to state that meanwhile the number of review articles dealing with EV as carrier/mediator/vehicle of information appears to exceed that of original research studies about some aspects of their appearance and structure. Most strikingly, the number of review articles propagating the information hypothesis is considerably higher than the number of experimental findings which demonstrate the transfer of biological information by EV in unequivocal fashion. What is the state of data-based knowledge concerning intercellular information transfer by EV?

The transfer of biological information (a typical metaphor from IT sciences adopted by life sciences in a rather unreflected way) in the only useful narrow meaning of the terms "transfer" and "information" implies the induction of the process of (re-)programming of a target cell by a donor cell for the adoption of a new phenotype or of one to several specific physiological function(s), that may be related or unrelated to that/those of the donor cells, with the help of messengers, such as molecules, waves or currents. Importantly, from a structural and functional point of view the messengers have nothing to do with the transferred information and the machinery of its decoding and realization by the target cells. Thus biological information has to be regarded as the operator (e.g. ligand) of a switch (e.g. receptor) controlling a physiological circuit (e.g. for growth, differentiation, metabolism), which is typically realized by a single entity (e.g. hormone, cytokine, ion, miRNA, light) and does not require a multi-component complex as its material/molecular basis, i.e. for pressing the button. The validity of this definition of the term "information" for the area of life sciences is exemplified best by the action of hormones, neurotransmitters or action potentials. For instance, the polypeptide hormone insulin is secreted by the pancreatic ß-cells (donor cells) in response to elevated blood glucose levels in order to "instruct" the adipose, muscle and liver (target) cells to take up glucose for storage as glycogen or triacylglycerol. Insulin apparently encodes the information "High Blood Glucose", recognized by the ß-cells, which subsequently becomes decoded by the insulin receptor of the target cells. Importantly, insulin as the information carrier is completely unrelated with regard to both structure (peptide) and mode of action (hormone which binds to a cognate receptor for its activation and tyrosine autophosphorylation) to the transferred information "High Blood Glucose" and the instruction for stimulation of glucose uptake and metabolism. In other words, the information carrier insulin as substance/molecule does not form and act as a component of the information decoding machinery of the glycogen and lipid biosynthetic pathways in the target cells. Insulin itself does not contribute to glucose and lipid metabolism, e.g. as glucose transporter or enzyme.

Can this definition of biological information transfer be regarded as being fulfilled for EV on basis of the experimental evidence presented so far? On basis of the currently available data, only a few studies have been devoted to demonstrate (patho)physiological effects exerted by isolated, purified and adequately characterized EV, which display the typical characteristics of sealed phospholipid membrane vesicles with embedded membrane proteins and enclosed mRNAs/microRNAs, rather than by the individual constituents such as miRNAs.[155] Among them are the seminal demonstrations that (i) a truncated constitutively active and oncogenic version of the epidermal growth factor, EGFRvIII, becomes transferred from tumor cells to normal cells via EV causing their malignant transformation,[113,114,122] (ii) EV released from glioblastoma cells transfer RNAs and proteins which foster tumor development[112] and (iii) the GPI-AP, Gce1 and CD73, together with the mRNA for GPAT3, which are involved in the control of lipolysis and lipogenesis, respectively, are transferred from (large) to (small) adipocytes via EV leading to upregulation of lipid synthesis in the latter (target) cells.[139,140,156] Importantly, in each of these cases, the EV actually transfer materials, i.e. molecular components, which following uptake are directly and stoichiometrically involved in the donor cell-instructed processes in the target cells, such as receptor downstream signaling for cell division by EGFRvIII or GPAT3-catalyzed lipid synthesis and its (c)AMP-dependent regulation by Gce1 and CD73. In other words, the transferred EV components EGFRvIII, GPAT3, Gce1, CD73 exert their authentic receptor and enzymic functions, respectively, rather than encode information, which in indirect and non-stoichiometric fashion presses the switches for oncogenic transformation and lipid storage, respectively.

On the basis of these considerations it is strongly recommended that the (patho)physiological role of EV should be interpreted as material transfer rather than information transfer as long as the experimental evidence available does not support the term information for the load of EV in the scientifically strict and only useful application. Moreover, the putative value of the concept of EV, in general, and of those equipped with GPI-AP, in particular, as novel "complex" biomarkers for the prediction, diagnosis, prognosis and therapy monitoring of common complex diseases critically relies on the expression of a multitude and variety of distinct physical materials by EV to be transferred from donor to target cells via body fluids for direct participation in multi-step pathways and/or in the coordination of distinct pathways. In contrast, the transfer of single information encoded by EV, such as for tumor transformation or lipid synthesis, does not necessitate (extensive) variation of the composition or structure of the corresponding EV, making them unattractive for use as biomarkers. This limitation is exemplified best by the rather low predictive value of typical information carriers, such as insulin and growth factors. In fact, the unambiguous demonstration of the compositional and/or structural diversity of EV released from disease-relevant cell types or tissues, in general, and of the association of certain compositional and structural subtypes/variants of EV with the development or state of a disease, in particular, has not been presented so far.

Finally, it is reasonable to assume that material transfer exerted by EV is physiological and efficient for short distances, only, in order (i) to guarantee its specificity with regard to the material's destination, (ii) to limit the waste of materials and energy for its production in case of broad distribution (*via* the circulation) over the organism and eventual failure of reaching the envisaged destination, (iii) to avoid deleterious effects caused by materials upon transfer to undesirable locations and eventual unforeseen uptake by non-target cells, (iv) to bypass the restrictions for the path from donor cells to acceptor cells in distinct tissue depots or organs *via* the circulation which depends on passage of the EV across vascular endothelial cells of the underlying vessels, possibly involving transcytosis or transient opening of tight junctions. In fact, the operation of a paracrine rather than endocrine mode of EV action was clearly reported for the adipose tissue depot.[157,158] EV, equipped with specific GPI-AP, mRNAs and miRNAs which are all engaged in triacylglycerol synthesis, become released from large adipocytes and then transferred to small neighboring adipocytes for uptake and direct participation in lipid synthesis. This apparent paracrine material transfer was interpreted as shift of the burden of lipid loading from large (filled-up) adipocytes to small (empty) adipocytes through the coordination of the triacylglycerol synthesizing capacity within a tissue depot.[159] A paracrine transfer of exosomal miRNA was also reported between T-cells and antigen-presenting cells within immune organs[160] and of both mRNAs and proteins between embryonic stem cells and blood cell progenitors within hematopoietic tissues.[161]

At variance, during certain pathophysiological situations, such as malignant transformation, "spill-over" of EV from malignant tissues into the blood may lead to the distribution and subsequent transfer of oncogenic materials to hitherto benign tissue cells. This would explain the very low number of studies reported so far, which provide unequivocal evidence for the presence of tissue-derived EV in mammalian plasma. No doubt, the major portion of plasma EV is derived from blood cells. This distribution ratio, i.e. the (presumably) very low concentration of tissue-derived EV, presumably reduces the usefulness of plasma EV as biomarkers for (common complex) diseases, which are caused by functional impairment of tissue cells. However, at present systemic appearance and function of EV cannot be ruled out completely. One of the rare experimental studies addressing this issue demonstrated the fusion of tissue-factor-bearing microvesicles with activated platelets and the accompanying initiation of coagulation.[162]

GLEC-associated GPI-AP as biomarkers

"Reductionistic" approaches for the identification of novel biomarkers are based on the measurement of the levels of a single or a few predominant and defined protein, lipid or metabolite species (e.g. by PCR or ELISA methods). They provide the complete but biased understanding of the only and seemingly linear metabolic pathway thought to be responsible according to a working hypothesis, which is typically based on text book knowledge. In contrast, "holistic" approaches rely on the determination of changes in the levels and fluxes of all relevant components amenable to untargeted "Omics" technologies and lead to the complete but unbiased understanding of the underlying network of interacting pathways without focusing on a specific one. Unfortunately, so far both approaches have demonstrated only limited predictive power for T2D, which does not support stratification and individualized therapy. This failure may, in part, be due to the limited information depth intrinsic to both "reductionism" and "holism" which is presumably caused by loss of the "interactome" and of (biophysical) properties intrinsic to macromolecular complexes, respectively.A novel "hermeneutic phenomenological" approach may lead to biomarkers of higher predictive power reflecting the intimate interplay between susceptibility genes and environmental cues in a more direct and precise fashion than previous "reductionistic" and "holistic" approaches. It relies on the demonstration and biophysical characterization of extracellular complexes in plasma, which harbor GPI-AP and phospholipids and possibly additional proteinaceous (e.g. transmembrane proteins) and lipidic (e.g. cholesterol) components in micelle-like configuration, the so-called GLEC (see above), and may be released from almost each cell type through non-classical secretory mechanisms. The minimal bias of this "hermeneutic phenomenological" approach is the assumption that GPI-AP with complete GPI anchor are susceptible for release from cells into GLEC, which relies on the following rationale: (i) GPI-AP may be particularly prone to spontaneous or regulated release from the cell surface due to sole anchorage at the extracellular leaflet of the plasma membrane phospholipid bilayer via their covalently attached GPI moiety. (ii) This release of the amphiphilic GPI-AP equipped with the complete GPI moiety (in contrast to that of the soluble GPI-AP as a consequence of lipolytic or proteolytic cleavage of the GPI or protein moiety, respectively) necessitates its embedding into amphiphilic structures, such as surfactant- or lipoprotein-like particles, nodal vesicular particles, milk fat globules, exosomes or microvesicles, but also GLEC. (iii) The rate of the release of GLEC from the surface of metabolically relevant cells into the circulation and/or their morphological and biophysical characteristics are correlated to (metabolic) stress, exerted by chemical and mechanical factors (such as elevated levels of plasma lipids, insulin or reactive oxygen species, shearing forces, cell deformation through compression or stretching), as is prevalent during early stages of many (metabolic) diseases, in general, and T2D pathogenesis, in particular. (iv) Gender-specific differences in the levels and the types of plasma GLEC detected by

chip-based sensing of serum from obese diabetic subjects are expected for both the prediction of T2D and its stratification into subtypes on basis of the known slightly, yet significantly increased probability for T2D in case of being descendant of a diabetic father and an unaffected mother compared to the inverse constellation as well as of minor, yet clinically relevant gender-specific differences in the manifestation of diabetic late complications.

The following experimental evidence is compatible with this rationale: (i) GLEC are released from metabolically relevant tissues (e.g. adipose, muscle, liver, ß-cells, endothelial cells) into the circulation in response to (metabolic) stress (e.g. high levels of glucose and fatty acids), as is prevalent during obesity and T2D, but also along other pathogenic developments. (ii) GLEC differ in level, type (structure, composition) and biophysical properties (viscoelasticity, rigidity) between distinct subtypes of frank T2D (prerequisite for biomarkers for disease stratification) and/or between obese subjects characterized by varying life styles, life stages and disease states along the pathogenesis of T2D (prerequisite for biomarkers for prediction). (iii) GLEC in serum can be detected and characterized with regard to level, type and biophysics using chip-based sensors (see below). (iv) Certain GPI-AP, such as CD73 and Gce1, have been reported to be released from metabolically relevant cell types, such as primary and cultured adipocytes, in response to metabolically relevant stress factors, such as high levels of saturated fatty acids, reactive oxygen species and anti-diabetic drugs.[134,135,163–168] Likewise, for a number of GPI-AP elevated levels have been measured in plasma from cancer patients.[68,69] (v) GPI-AP rather than typical transmembrane proteins are prone to rapid and efficient release from the plasma membranes of donor cells in course of mechanically or chemically induced stress. (vi) Phospholipids in complex with GPI-AP have been detected in the supernatants of cultured cells as well as in rodent and human serum and may represent constituents of micelle-like complexes such as GLEC, lipoprotein-like particles such as SLP, globule-like particles such as MFG, and EV such as exosomes. (vii) Plasma phospholipids analyzed by untargeted lipidomics have been shown to predict early neurodegeneration during preclinical and presymptomatic Alzheimer's disease,[169,170] that is presumably associated with metabolic disturbances.[171,172] (viii) GPI-AP are known to be susceptible for transfer from donor cells to acceptor cells *in vitro*[173] and *in vivo*[174,175] in a functional state, thereby putatively transmitting biological materials within or between tissues (e.g. CD73 as anti-inflammatory and immune-suppressive molecules). Interestingly, the level of the GPI-AP CD73 in plasma was shown to be correlated with insulin sensitivity in diabetic mice and human probands.[176-178] (ix) Phospholipids in complex with membrane proteins (e.g. caveolins) were reported to be released from vascular endothelial cells *in vitro* into culture medium and *in vivo* into plasma of mice following oxidative stress and high fat diet.[179-182] (x) The level of the GPI-AP CD73 in plasma was shown to be correlated with insulin sensitivity in diabetic mice and human probands.[183-185] (xi) Vesicle- and lipoprotein-like structures harboring GPI-AP have been identified in plasma.[23,32,33,186]

Together, those data documenting elevated plasma levels of GPI-AP indicate that GPI modification is a potentially useful biomarker for the detection of certain human diseases. In fact, a GPI-specific lectin was used previously as a diagnostic tool to detect GPI-AP in human plasma.[69] Elevated plasma levels of GPI-AP, possibly harboring the complete GPI anchor were measured for patients with ovarian cancer, glioblastoma brain tumors and lower grade colon adenocarcinomas, known to be exposed to metabolic stress as a consequence of their disease.

The study of GLEC-associated GPI-AP

The presence of GPI-AP displaying the complete GPI anchor and GLEC in body fluids of T2D patients has not been studied so far. Typical conventional and commonly used methods for the analysis of the classical secretome in serum (e.g. ELISA, Western blotting, 2D-PAGE, mass spectrometry) are biased towards the detection of predominantly high-abundance proteins. This necessitates complex and tedious fractionation procedures for the enrichment of low-abundance components, such as GLEC. However, those methods most likely will fail for the analysis of GLEC for the following reasons: (i) Loss in course of sample preparation by centrifugation or flotation; (ii) disrupture in course of sample solubilization for SDS-PAGE; (iii) inadequate sensitivity and resolution; (iv) inadequate throughput due to time-consuming procedures for enrichment.

To overcome these hurdles, a chip- and microfluidic channel-based sensor has been developped recently with the objectives of specific detection and biophysical characterization of large macromolecules, such as GLEC, even in the presence of complex matrices, such as serum and hydrophobic agents, such as phospholipids. It relies on the generation of surface acoustic waves (SAW) at the gold surface of chips equipped with microfluidic channels.[187–189] Any interaction of GLEC with the gold surface, which eventually is triggered by their capturing through alpha-toxin covalently coupled to the surface, will result in changes in the shape of the SAW, manifested in both rightward-shifts in their phase (i.e. declines in frequency) and reductions in their amplitude (Figure 1). The major advantages of the SAW *vs*. the commonly used surface plasmon resonance sensor rely on the potential to measure large (lipid-containing) macromolecules even in the presence of turbid matrices, such as serum[188,190,191] as well as on the high sensitivity towards putative alterations in the composition (proteins, phospholipids) and structure of the GLEC. Albeit chip-based sensing *per se* does not enable the delineation of the type of GLEC contained in a given sample, the SAW signature will provide a summation signal which is characteristic for the sample GLEC "in total".[71,192–195]

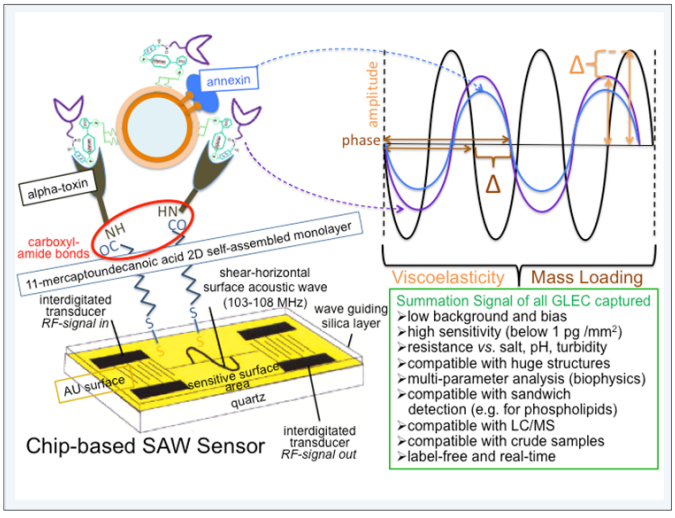

Figure 1 The principle of chip-based SAW sensing of serum GLEC.

The specific capturing of the GLEC by the SAW chip-based sensor is accomplished by the GPI-binding protein α-toxin upon its covalent coupling to the chip surface. GPI-AP act as receptor for the bacterial α-toxin of *Clostridium septicum*. Ample evidence has accumulated during the past decade that the highly conserved glycan core of the GPI anchor is the major binding determinant for α-toxin. Nevertheless, it cannot be excluded at present that GPI anchors of different GPI-AP may differ in their relative affinity for α-toxin to a limited extent, which could be due to structural variation of the protein moiety of the GPI-AP. The covalent coupling of α-toxin to the gold surface of long-chain 2D self-assembled CM-dextran monolayer chips was performed using the conventional EDC/NHS-based protocol and monitored by measuring the phase shift in course of the reaction. The signals generated by the sensor and recorded in real-time reflect the loading of mass onto the chip surface and, in addition, depend on the (bio-)physical properties of the contacting sample fluid, including and predominantly its viscoelasticity. Any covalent (e.g. coupling of α-toxin) or secondary (e.g. capturing of GPI-AP) interaction of molecules or complexes with the chip surface will lead to right-ward shifts in phase and/or reductions in amplitude of the shear-horizontal SAW propagating along the chip surface. Those changes reflect alterations in mass loading and/or viscoelasticity, respectively, exerted by the interacting materials. The nature of the GLEC was shown by detection of phospholipids in course of sequential binding "in sandwich" of the Ca^{2+}-dependent phospholipid- (i.e. phosphatidylserine)-sequestering protein annexin-V (in the presence of Ca^{2+}). The sequential right-ward shifts in phase (mass loading of GLEC and annexin-V) and reductions in amplitude provoked by capturing of GLEC and annexin-V (blue curves) *vs.* control chips (black curves) are indicated by (hatched, brown and orange) arrows and (brown and orange) triangles, respectively. The advantages of the chip-based SAW sensing resulting in a summation signal for all GLEC captured are summarized in the green rectangle.

Conclusion

GPI anchors have been attributed a variety of (patho) physiologically relevant functions and features in addition to mere membrane anchorage of cell surface proteins. These encompass the lateral membrane mobility and packing density of cell surface proteins, which are both elevated compared to transmembrane proteins. Furthermore, the susceptibility of GPI-AP for lipolytic cleavage of their anchor structure and the tissue-specific expression of (G)PI-specific PLC/D strongly suggest that the release of GPI-AP from cellular membranes may be a (patho)physiologically regulated and controlled event. However, a potential role of lipolytic GPI anchor cleavage in cellular signaling has proved to be more controversial. GPI anchors and fragments derived from the GPI glycan core have been implicated as mediators of insulin action, but the potency, the precise molecular structure and the cellular origin of those postulated messenger molecules have remained elusive and prevented their use as disease biomarkers. During the past two decades another putative function of GPI-AP, which could also explain the reason why during evolution the expression of certain cell surface proteins as GPI-AP had been introduced rather than as typical bi- or polytopic transmembrane protein, has been the object of intense research efforts. This refers to the release of GPI-AP from the extracellular face of the plasma membranes without (proteolytic or lipolytic) cleavage of the anchor moiety, which instead remains attached to the protein moiety in completion. Albeit a number of GPI-AP have meanwhile been identified which can be recovered from extracellular aqueous compartments, such as body fluids, and are equipped with the complete GPI anchor, the underlying molecular structures and mechanisms and the (patho)physiological processes mediated thereby remain to be elucidated as well as a putative biomedical application for the released GPI-AP. With regard to the latter, it is conceivable that GPI-AP assembled into GLEC could serve as biomarkers for the prediction, diagnosis, prognosis and stratification of common complex diseases. Certainly, the value of a predictive biomarker increases with (i) the time point of its initial appearance and first possibility of its technical measurement prior to disease onset, (ii) the detection in easily accessible body fluids, such as serum, and (iii) the detectable differences between individuals with regard to lifeworld, disease onset and disease outcome. These criteria may be fulfilled by novel "phenomenological" biomarkers, such as GLEC for the prediction of T2D.

Strikingly, the release of GPI-AP together with the complete GPI anchor could be used as pilot project study to demonstrate the successful transformation of a biochemical phenomenon to biomedical meaning, such as use as biomarkers for common complex diseases, without the need to acquire molecular, mechanistic and causal "wisdom" as a result of canonic hypothesis-driven "reductionistic" research. At variance, a "hermeneutic phenomenological approach" relying on (i) mere description and correlative analysis on the basis of a minimal and well-recognized (and accepted) bias or (scientific) tradition, (ii) presentation of the findings as database rather than as (non-fictional) narrative in the typical neutral and irrefutable style of a scientific publication and (iii) very moderate interpretation leading to an understanding rather than to explanations and lacking theories and models according to the credo of "the end of theory":[196] "Correlation is enough"...and..."supersedes causation, and science can advance even without coherent models, unified theories, or really any mechanistic explanation at all."

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Conflicts of interest

The author declares there is no conflict of interest.

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