Extracellular Vesicle-based biomarker discovery: opportunities and challenges in the –post-omic era

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ABSTRACT (100/100 Words)

The research on extracellular vesicles (EVs) has been exponentially rising during the past few years. EVs are found in different biofluids and are potential sources for the discovery of novel biomarkers. In this review, we demonstrate a conceptual overview of the field and current knowledge, critically assess the new advances in the field of EV biomarkers, and discuss different challenges to validate and implement EVs as clinical diagnostics. Future implementation of 'omics-based technologies and integration of systems biology approaches for the development of EV-based biomarkers and personalized medicine are also considered.

Extracellular vesicles (EV) were historically considered to be membrane-derived cellular debris with no biological or clinical significance produced during cell death. However, evidence is amassing that EVs can exert multiple physiological and pathological functions as important mediators of intercellular communications [1-3]. Thus, such particles have been isolated from almost all cell types, mucosal and endogenous biofluids (blood, urine, saliva, cerebrospinal fluid, lymph, etc.) and have been implicated in key processes such as growth and development, cell-to-cell communication, immunomodulation, blood coagulation, and various stages of tumorigenesis [4-8]. **Table 1** and **Table 2** exemplify EVs isolated from different cellular sources and biofluids.

Diverse molecular cargoes have been recovered from EVs, such as nucleic acids, proteins, and lipids. Notably, these cargoes appear to be protected against degrading enzymes such as nucleases and proteases: protection is afforded by a natural lipid bilayer capsule derived from the cytosolic membrane of the originating cell shedding the EV [1,9-12]. Importantly, much like synthetic liposomal micro and nanoparticles, the EV lipid bilayer and its enclosed cargo are stable under physicochemical conditions generally considered adverse for biological materials, such as long-term storage, multiple freeze-thaw cycles, and extreme pH [13]. Several groups have shown that pathological states such as oxidative stress, transformation, apoptosis, and ethanol-induced cell injury induce cells to increase their EV release rate, simultaneously altering their composition to reflect the altered state of the cellular origin [3,14-18]. Together, these characteristics position EVs as a new, highly appealing class of biomarkers with strong diagnostic potential in the context of personalized medicine [19,20]. This review explores in depth the potential of EVs as biomarkers of clinical utility. Current knowledge of EV subtypes, their biogenesis, and pathophysiological role are outlined, with emphasis placed on advantages against competing

analytes. Challenges to achieving the diagnostic potential of EVs including sample handling, EV isolation, method standardization, and bioassay reproducibility are discussed. These features will be presented in the context of systems biology and personalized medicine, two relatively new but rapidly expanding and unifying fields relevant to therapeutics innovation.

Extracellular vesicles: subtypes and mechanisms of biogenesis

The term 'EV' collectively refers to a heterogeneous vesicular population spanning 50 to 10,000 nm in size **(Box1).** Distinct subpopulations include exosomes, microvesicles/microparticles, and apoptotic bodies [21,22], although the terms not associated with cell death have been used interchangeably in the past[16, 34]. These EVs are secreted from almost all cell types into the aqueous extracellular microenvironment and represent a snapshot of the cell status at the time of release, as defined by their components [3,23]. Beyond size, which is itself inadequate [24], morphological characteristics such as density, mode of biogenesis, and molecular markers such as CD63, CD81 and Annexin V are used to classify EVs as outlined in Box1.

Exosomes are the smallest (30 nm-100 nm) and most heavily studied subpopulation of EVs [25,26]. These particles are generated by the exocytosis of multivesicular bodies (MVBs) [27,28] (**Figure 1**). Early endosomes can be targeted for ubiquitin-dependent interactions with one of three endosomal sorting complexes required for transport (ESCRT-0, ESCRT-I and ESCRT-II), leading to recycling of the endosome or, alternatively, its progression towards a late endosomal pathway [29]. Late endosomal pathways are dependent on MVBs, are ubiquitin-independent, and lead to the formation and sorting of exosomes [30]. In this pathway, ALIX (ALG-2-interacting protein X) binding to the exosomal cargo molecules, can drive MVB to go through exosomal sorting pathways instead of lysosomal recycling pathways [31]. While ESCRT complexes are

clearly required for the lysosomal degradation of proteins, their contribution to exosome formation is less well studied [32]. However, selected ESCRT components and accessory proteins such as Signal Transducing Adaptor Molecule 1(STAM1), Tumor Susceptibility 101(TSG101), and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) are involved in exosome biogenesis machinery [33]. The Rab GTPase family regulates fusion of late-endosomal MVBs with the plasma membrane and exosome release [34]. Thus, Rab5 and Rab7 regulate endocytic trafficking downstream of MVB biogenesis and cargo sequestration whereas Rab27a, Rab27b, and Rab35 control exosome secretion [31,34,35].

Microvesicles (also called shedding vesicles, shedding microvesicles, or microparticles) are approximately 100-1000 nm in diameter and originate from the outward budding of the plasma membrane [21]. The protein TSG101, which is also involved in exosome biogenesis, interacts with arrestin domain-containing protein 1 in the budding stage of microvesicles [36]. The release of both exosomes and microvesicles is associated with a specific region of the plasma membrane enriched in cholesterol, lipid rafts, and ceramide [13,37-39]. Unlike endosome maturation, the small GTP-binding protein ARF6, the Rho signaling pathway, actin motors, and elements of the cytoskeleton are involved in the formation of microvesicles [40-42].

Apoptotic vesicles are a subpopulation of EVs that range from 100-2000 nm in diameter and are generated by the blebbing of plasma membrane of cells undergoing apoptosis. Larger apoptotic vesicles (1000-5000 nm) are referred to as apoptotic bodies and contain fragmented nuclei as well as fragmented cytoplasmic organelles [6,7]. Importantly, the uptake of apoptotic bodies originating from tumor cells can transfer oncogenic contents to the recipient cells [18]. For example, apoptotic bodies derived from rat embryonic fibroblasts carrying H-ras^{V12} and human c-myc oncogenes were internalized by recipient cells (mouse embryonic fibroblasts), resulting in

loss of contact inhibition *in vitro* and tumorigenic phenotype *in vivo*. Fluorescence *in situ* hybridization analysis, revealed the presence of rat and mouse fusion chromosomes in the nuclei of the recipient murine cells, propagation of aneuploidy and the accumulation of genetic changes necessary for tumor formation [18].

The classification of extracellular vesicles can be based on size, density, protein composition, and cell specific markers [43,44]. One of these parameters may not be adequate alone, however, as, for example, vesicles originating from different biogenesis pathways might have overlapping diameter ranges [24]. Thus, the terminology referring to exosomes and extracellular vesicles has changed substantially over the past decade and the words "exosomes", "microvesicles", and "microparticles" have been used interchangeably in the past [21,43]. Gaining a better understanding of vesicle formation led to the characterization of extracellular vesicles based on mode of origin. Nevertheless, current understanding of EV biogenesis is incomplete and further confounded by inconsistencies in EV isolation and characterization protocols. However, the identification of vesicles by specific molecular patterns, is expected to progressively deepen characterization of these biological products under refined, universally agreed criteria will lead to more advanced models of EV classification. The presently understood roles of EVs in the pathogenesis of disease is summarized in **Table 3**.

EVs as biomarkers: promises and pitfalls

The National Institutes of Health define the term biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [45]. In this context, the data deluge

obtained through comprehensive profiling of the genome, transcriptome, proteome, and metabolome in health and disease has catalyzed a paradigm shift on how basic biomedical research is conducted. Crucially, these data have repeatedly hinted at the potential for early, accurate disease diagnosis with high sensitivity. Personalized disease progression monitoring might also be achievable through identification or measurement of one or more of these biomarker classes- so called 'biomarker signatures'. In this respect, the promise of EVs is growing exponentially **(Figure 2).**

Liquid biopsy for EV sampling offers a number of advantages over other diagnostic methods: Firstly, overall EV levels are usually elevated in disease – a finding that has been proposed as a simple measurement tool, but also engendered skepticism over disease-specific value. Thus, a 'general stress signal' view is adopted by many [6,15,46,47]. However, repeated evidence has emerged of EV enrichment with specific molecular components (RNAs, proteins, and lipids) that reflect the status of the parental cell; in many, but not all cases, these biomarkers are enriched in the EVs in a disease-specific manner [47-50]. These findings have motivated research beyond disease correlation into establishment of causality: thus EVs might represent not only robust vehicles of disease-specific biomarkers, but therapeutic targets in themselves.

Secondly, the lipid bilayer of EVs contributes further to diagnostic utility by protecting biomacromolecules and stabilizing them from RNases, proteinases, and other enzymatic activity present in the biofluids. For example, in bovine milk, naturally existing miRNA and mRNA which were associated with EVs were shown to be resistant to adverse acidic conditions (treated for 1 h in an acidic (pH 1) solution) and RNase treatment while synthetic spiked-in miRNAs were prone to degradation under similar conditions [51,52]. Several other reports showed that the total yield of EV RNA is not significantly changed after treatment with RNase, irrespective of

EV origin: cell culture media, serum, or plasma [9,11,12,23]. Thus EVs are very stable allowing storage for an extended period of time, in start contrast to many biomarker assays that require processing of fresh biofluids [53]. Furthermore, in a multiplex study on ovarian cancer patients which identified eight miRNAs for discrimination of ovarian cancer from benign ovarian disease, miRNA levels were not altered by pre-analytical variables such as collection and storage time [14]. Thus, analysis of biomarkers within the EV fraction of biofluids promises a potential solution against poor analyte stability and deviation from sample handling standard operating procedures (SOP), which are factors well known to confound the outcomes of plenty a clinical trial [24,47,54].

Thirdly, notwithstanding therapeutic relevance and sampling robustness, EV-based analysis offers a substantial statistical advantage in reducing biological matrix complexity and thereby overall assay noise. This significant improvement facilitates considerably more specific and sensitive detection of low abundance biomacromolecules [47,50,55] or analytes with varying levels within sub-compartments of a complex biological matrix. A simple analogy can be drawn with the value of cell sorting in diagnostic haematology[56], as well as *in vivo* pharmacology [57]. This is commonly referred to as the "less is more" principle: a smaller, but more defined sample is highly enriched for specific biomarkers during the exosomal sorting and isolation process, that would otherwise constitute only a very small proportion (less than 0.01% v/v) of an unprocessed biofluid sample [58]. Thus, several studies reported increased sensitivity for EV-based biomarkers compared to whole serum and urine biomarkers [59-61]. For instance, miRNA found in EVs isolated from sera of patients with colorectal cancers, showed higher sensitivity (90%) compared to serum biomarkers CEA and CA19-9 (30.7 and 16% respectively) [59]. In several studies, higher levels of disease-specific biomarkers were found in the EV-enriched fraction of biofluids

compared to the EV-depleted fraction [61,62]. For example, a miRNA subset enriched in EVs isolated from the serum of prostate cancer patients was hardly detectable in healthy subjects. In stark contrast, the most abundant miRNAs in EV depleted sera were recovered from both healthy and prostate cancer subjects[61]. Similarly, in alcoholic hepatitis, serum/plasma miRNA-122 and miRNA-155 levels were correlated with liver damage and were predominantly associated with the exosome-rich fraction (around 5 times more) compared to the non-exosome fraction [62].

Consequently, the potential value of EVs has not escaped the attention of the personalized medicine community [20,63,64]. However, there remain significant challenges in the commercialization of such approaches beyond centralized, specialist laboratories and especially in diagnostic kit format. Thus, whilst the US FDA enables regulated (CLIA) laboratories to carry out so-called 'homebrew' tests, including on fractions of samples such as EVs, marketing of such diagnostic tests in kit format requires up to phase III clinical trials and regulatory approval in line with the standards for reporting diagnostic accuracy (STARD) [65]. In Europe regulations are presently less stringent, through the self-certification CE marking scheme, but this is expected to align closer with FDA standards within the coming 5-10 years [66]. Recent changes to this framework comes in the form of the FDA's *de novo* regulatory path for diagnostics development. This route has been opened to enable new analytical methodologies with no previous golden standard, such as whole genome sequencing, to reach the market. However, as evidenced through several instances of FDA intervention (e.g. Theranos), sample processing, wherein EV enrichment falls, is considered a separate step to analyte measurement technology in the diagnostic SOP continuum. Thus, commercialization of EV enrichment technologies is expected to follow the 510k FDA pathway, requiring high levels of clinical rigor and validation ahead of diagnostic use marketing. This will most probably be achieved with enrichment device/process

alignment with one or more innovative biomarkers, whose commercial value pivots on the SOP, stability, statistical, and the 'less is more' principles cornerstone to the utility of EVs for biomarker recovery. Thus, the predicted EV biomarker workflow is depicted in **Figure 3.**

Challenges for Standardization of EV biomarker discovery

Despite the great interest in the role of EVs in different pathological conditions, there exist important limitations at the pre-analytical, isolation, characterization, biological, clinical and post analytical levels. Together, these contribute to confound concordance between different studies and raise questions with regards to ultimate clinical value. In line with the value of standardization in other separation-based diagnostic technologies, establishing an efficient, rapid and reproducible isolation method is crucial to analytical reproducibility. In the last few years a variety of EV isolation technologies have been developed with each technique providing specific advantages and disadvantages to downstream analytics. Consequently, the lack of standardization hinders the translational process. Validation studies pivot on the systematic, orthogonal transfer of methods from research to development, in a manner that is end user-friendly and as simplified as possible. Indeed, many of the presently available methods are inherently prone to variance or poorly suited to standardization for the diagnostic laboratory setting.

EV isolation methods

Although EVs have been successfully isolated from different biofluids, including CSF [67], plasma [68], urine [69], serum [3], saliva [70], amniotic fluid [71], and breast milk [72], the enrichment methods have included ultracentrifugation, antibody-coated magnetic beads, microfluidic devices, polymeric precipitation technologies, size exclusion, sieving, porous nano-structures, and other new

technologies **(Table 4).** Yet the impact of sample processing on study outcome is nothing new [21] and extends the paradigms of confounding factors reported for various proteomics and transcriptomic methods, even metagenomics and microbiomic studies[73]. To date, none of these reported methods has been shown to offer consistent superiority, whether by diagnostic purpose, type of biofluid, EV subclass or clinical setting. Indeed, either one or a combination of these methods might be used. Selection of a preferred method is greatly dependent on the goal to be achieved as well as preconceptions, assumptions and individual laboratory habits. In general, researchers aim for high EV purity and yield, either at the whole population of EVs or a subclass (exosomes, microvesicles, apoptotic bodies). The choice, however, is based on concurrent trends for the target disease, perhaps specific mechanistic interests in the underlying pathology, but principally on grounds of resource intensity (cost effectiveness against process complexity). However, preferential aim tradeoffs should be made depending on the circumstances, with hypothesis-driven mechanistic considerations at the center of process selection in order to maximize effectiveness.

Ultracentrifugation

Traditionally, the gold standard and most commonly used protocol for EV isolation/purification is differential centrifugation, which involves multiple centrifugation and ultracentrifugation steps. As protocols vary between users, this may lead to inconsistencies in the recovery of EVs. In general, the centrifugation protocol starts with a low speed centrifugation (300-500 g for 10-15 min) to pellet cells, followed by a medium speed (10,000 to 20,000 g for 20 min) to eliminate larger vesicles and a final 100,000 g ultracentrifugation step for >2 h to pellet EVs. The protocol should be optimized based on important factors such as viscosity[74] and rotor type (k factor)[77].

Thus, in conditions that alter sedimentation rate, or diseases that increase the viscosity of biofluids such as hyperviscosity IgM syndromes, cryoglobulinaemia, and macroglobulinaemia, the dilution of biofluids prior to ultracentrifugation should be considered [24,75]. Similarly, the rotor k-factor is a commonly ignored parameter that, nonetheless, underpins the rotor efficiency in pelleting particles. Briefly, the value of the k-factor is determined by the maximum angular velocity (ω) of a centrifuge (in rad/s) and the minimum and maximum radius (r) of the rotor [21,76]. Consequently, yhe k-factor can influence the purity and yield of exosomes in the ultracentifugation steps and can be utilized to predict the time required for achieving the desired sedimentation profile.

Last but not least, the substantial risk of co-precipitation viruses and protein aggregates should be considered where such particles are within the EV size range assayed [21]. Often this is ignored in some protocols, where the first and second centrifugation steps are replaced by faster, higher purity microfiltration techniques [78]. To compensate and minimise such carry-over issues an extra purification step can be added after the last centrifugation step, such as sucrose gradient or immunomagnetic isolation [21,23].

Size exclusion techniques

Size exclusion techniques, including ultrafiltration and chromatography, are a rapid and inexpensive alternative solution[79,80]. Although these methods can accommodate a large volume of biofluids, they are unable to concentrate EVs, selectively isolate subpopulations and, in the case of ultrafiltration, may cause deformation of large vesicles [24]. However, nanomembrane ultrafiltration concentrators such as the Millipore Centricon™ and Amicon® Ultra-15 Centrifugal Filter Devices can be used to simultaneously concentrate and filter the samples [9].

Immune affinity isolation

Immune isolation can increase EV purity and enable selective capture of specific subpopulations based on one or more surface markers. In this approach, antibodies against defined surface protein markers are used conjugated/coated on beads. The captured EVs are then typically separated using magnetic-bead principles [21]. The approach is versatile method and compatible with downstream analysis including western blotting, flow cytometry, electron microscopy and transcriptomics[23]. Beyond antibodies, other affinity-based methods include synthetic peptides such as venceremin (Vn) that exhibit specific affinity for canonical heat shock proteins[81], as well as target-specific, synthetic, single-stranded oligonucleotides (aptamers)[82].

Polymeric precipitation

Some newly introduced isolation techniques such as the ExoQuick™ (System Biosciences), Exosome Isolation Kit (Life Technologies) and ExoSpin Exosome Purification Kit (Cell Guidance Systems) can facilitate sedimentation of EVs from solution during low speed centrifugation (10,000–20,000 g) by promoting the precipitation of vesicles with polyethylene glycol or other polymers. Although these kits are faster and more efficient than ultracentrifugation, they also precipitate protein aggregates and lipoproteins. Interestingly, it has been demonstrated that a combination of polymeric precipitation methods, followed by immune affinity isolation against e.g. the exosomal marker CD63 can lead to a high yield of pure exosome subpopulations [9]. Given the success of combinatorial solutions, and the drive for SOP simplification, several new material engineering-based technologies have recently been introduced. However, clinical and comparative studies on their reproducibility and clinical efficiencies are limited.

Comparative methodology studies

The methods outlined above present a variety of physicochemical and biochemical means through which EV characterization can be approached. These have the potential to impact significantly on the constituents of the resulting processed sample matrix, thereby complicating the elucidation of the functional role of EVs, biomarker discovery efforts and targeted analytical assay development. Despite worldwide interest in this research area, only a limited number of comparative studies have been published in the literature to date.

Thus, a recent study investigating comparatively the expression profile of 375 miRNAs in EVs isolated from the sera of healthy individuals, either by ultracentrifugation or by polymer precipitation methods, reported that differences in the observed miRNA profile of EVs can be affected by the isolation method [83]. Elsewhere, 100 nm-liposomes fabricated with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were used as a model system to assess the effect of isolation protocols on EV recovery and size distribution[84]. Among the four different purification protocols evaluated, ExoSpin, Invitrogen kits, PureExo, and ultracentrifugation, the first two achieved up to 2 orders of magnitude higher EV yields. However, the authors did not characterize the recovered EVs based on surface markers or physicochemical methods such as dynamic light scattering and tunable resistive pulse sensing [84]. Van Deun *et al*., evaluated the role of different isolation protocols in downstream 'omics approaches for biomarker discovery. Density gradient centrifugation (Optiprep) yielded purer CD-63 positive EV fractions with less contaminating proteins such as Argonaute-2 (Ago2) complexes [85]. Amongst other functions, Ago2 is a member of the RNA-induced silencing (RISC) complex and is considered an extracellular RNA-binding protein which is associated with the

non-EV related fraction[86]. This preparation revealed a unique mRNA profile enriched for translation machinery and ribosomal proteins [85]. In a study conducted by Alvarez *et al.,* ultracentrifugation (traditional protocol, in combination with filtration or sucrose cushion) was compared to two other precipitation-based methods (ExoQuick-TC, System BioSciences) [87]. The authors modified the ExoQuick protocol and increased final centrifugation speed to 10,000 g, instead of the 1,500 g recommended by the manufacturer. Maximal EV, miRNA, and mRNA yield was obtained using the modified exosome precipitation protocol and RNA quality was suitable for downstream profiling. Similarly, Bukong *et al.*, compared ultracentrifugation to the ExoQuick precipitation kit ahead of immune affinity isolation against CD63 [23]. Although ExoQuick outperformed ultracentrifugation in terms of EV recovery, both methods resulted in very high purity, verified by the abundance of EVs and lack of protein aggregates in transmission electron microscopy images. Moreover, western blotting showed high yield of exosomal marker, CD63, after isolation with both methods. Such a combined precipitation and immune affinity protocol was found to be well-suited for use in the clinic in terms of simplicity, speed and sample throughput.

Notwithstanding the impact of EV isolation methodology, protein or RNA isolation methods may also impact upon the outcome of downstream analytics. Thus, EV RNA patterns were reported to vary in size and composition after isolation using different methods [88]. Mouse MC/9 cells were cultured and EVs were isolated by ultracentrifugation. RNA was isolated from EVs using seven commercially available RNA isolation kits. Generally, column-based methods were reported to outperform phenol-only or combined phenol and column approaches in terms of RNA yield and the highest yield was achieved by miRCURY^{TM} RNA with the mean of 21.8µg versus 6.1µg for Trizol. These studies clearly demonstrate a growing, and largely unmet need for

standardization and validation in EV sample preparation. Although precipitation methods are amenable to rapid, highly scalable, and effective EV isolation, clinical protocols require careful consideration of the research question. Furthermore, assay migration between methods should be supported by well-controlled studies leading to verification of successful implementation in the clinic prior to use. Researchers and clinicians should pay special consideration to the type of target biofluid as well as the type of biomarker, as different methodologies might be better suited for alternative matrices and analytes to those commonly used by a research group [89,90]. Readers are thus advised to implement appropriate comparative and confirmatory protocols as part of their pilot work in preparation of large scale studies.

Clinical, biological, and analytical challenges of EVs as biomarkers

The methodological variance in the EV biomarker research continuum is further augmented through additional clinical, biological, and analytical challenges **(**Table 5**).** In the absence of proper study design and normative controls it is impossible to partition biological variation from technical variation. It has thus been showed that demographic variables such as age, gender, and ethnicity can affect EV signatures [91-93]; yet control groups over age, gender, and ethnicity common to other clinical research is largely lacking in EV-targeted studies [24,92]. Alternatively, pooled healthy subject samples can be used as a control [94]. Elsewhere, repeated sampling of the same patient in the course of disease has been used to determine the variability of a specific EV-associated biomarker [24]. Other factors such as physical activity and diet have also been shown to affect EV release patterns and cargo; these are additional parameters that should be considered in study design or be adjusted for [95,96].

Furthermore, as with other biomarker technologies, protocol standardization and consistency throughout sample acquisition, storage, and processing should be aimed for.

A major challenge in EV-based diagnostics is the complexity of EV secretion mechanisms in different pathological conditions which is intertwined with the activation of a complex network of diverse, cross-talking molecular pathways with adaptive feedback loops. The biomarker field has shown repeatedly that patient stratification and better disease discrimination can be achieved through the use of more than one single marker of disease. Indeed, there are examples, including in the field of EV research, where combined analysis of different classes of analytes can substantially improve sensitivity and specificity [61]. Thus, Madhavan *et al*. reported an increase of pancreatic cancer-initiating cell protein markers including CD44v6, Tspan8, EpCAM, MET and CD104 as well as increases in the levels of miRNA-1246, miRNA-4644, miRNA-3976, and miRNA-4306 in the serum-exosomes of pancreatic cancer patients compared to patients with chronic pancreatitis, benign pancreatic tumor and healthy controls [61]. Crucially, combined measurement of proteins and exosomal miRNA in discriminating pancreatic cancer from other type of pancreatic diseases and health increased sensitivity up to 1.00 (CI:0.95-1.00) compared to protein analysis (0.96, CI: 0.88-0.99) or miRNA analysis (0.81, CI:0.71-0.89). The specificity for the combined approach was reported to be 0.80 (CI:0.67-0.90). These results make a strong case for signature biomarkers to transcend analyte classes.

Indeed, such an approach might broaden the knowledge and shed new light into processes through detection of previously overlooked factors and/or systems associated with systemic disease. For example, in a study of EV biomarkers in alcoholic hepatitis using chronic alcohol-

fed mice, in addition to miRNAs specific to liver (miRNA-192 and miRNA-122), the organ principally affected by ethanol intake, miRNAs specific to other organs including the heart were also detected [3]. It is presently unclear whether alcohol is causal or consequential to the observed impact on the heart, however such outcomes can help initiate illuminating follow up studies. Notably, where causality between biomarker and condition is not been established, so-called 'association' biomarkers can be considered confounders, might have limited validity in disease diagnosis and, crucially, lead to misinterpretation of data pertinent to the success of therapeutic interventions [97]. Yet evidence is emerging that the biomaterials directed into EVs under conditions such as alcoholic stress are highly regulated and do not always mirror fully the functional status of the originating cell. Thus, Saha *et al.* [98], reported that both miRNA-146 and miRNA-27a are significantly elevated in monocytes after ethanol exposure. However, only levels miRNA-27a appeared to be directed to EVs. Thus the interpretation of specific EV signatures should be anchored across the domains of correlational and causal biomarkers. Furthermore, variability across studies may assist in identifying associational biomarkers, which are more prone to bias and confounders.

In addition, difference in EV subtypes [99], secretion mechanisms, and cargo changes in various stages of disease [100] must be taken into consideration. For example, Ji *et al.* [99], showed that a colon cancer carcinoma cell line (LIM1863) released two distinct subtypes of exosomes, enriched in apical surface sorting proteins or basolateral surface sorting protein. Deep sequencing and proteomic analysis of the two subpopulations showed distinct miRNA and proteome profiles [99,101]. Thus, *in vivo* and patient observations need to be supported by parallel evaluations in cell lines, tissue culture and primary cell research, with particular attention to mechanistic detail in recapitulating the organism milieu. These efforts will help elucidate the role

of an EV release event in disease and inform the likelihood of recovery from a given biological matrix.

Research 'omics and the post-'omic clinical era: the example of RNA and EVs.

Accurate measurement of biomarkers, be they EV preparations or otherwise, pivots on analytical platform limit of detection, dynamic range, and the capacity of current technologies to comprehensively identify, interpret and manage the resulting data. Moreover, there is a critical need for transparency and reproducibility in the pipeline of biomarker discovery, including patient recruitment, data gathering, and processing – the so called open science model. The approach presents a unique proposition in fundamentally altering our approach to unlocking mechanisms of disease and disrupting patient care.

New advances in high-throughput technologies have ushered in the era of 'omics science- the simultaneous agnostic survey of tens to millions of biomarkers including, transcriptomics, proteomics, lipidomics and metabolomics [102,103]. Although 'omic technologies have been utilized in EV studies, the amount of literature is limited. Yet disease development is a complex process; inherited susceptibility and different environmental exposures can modulate disease risk and progression in an individual over time. These introduce dynamic interactions in the evolution of individual molecular mechanisms of disease initiation and progression. For example, the cancer genesis process is presently understood to be characterized by stochastic accumulation of mutations and dynamic evolution of clones. Thus, whole genome instability measurements and genome-based cell population heterogeneity have been linked to stages of cancer development [104,105]. Currently, most cancer biomarkers do not reflect the evolutionary dynamic of cancer progression but rather focus on specific deregulated pathways. Development of biomarkers for

cancer risk management should consider these stochastic and dynamic properties over time in neoplastic evolution. This approach is fundamentally different from the commonly used three-stage disease category of normal, disease without symptoms, and disease with symptoms model for biomarker screening. In fact, using stochastic modeling might provide a framework for guiding future biomarker research to enable more accurate patient stratification into various risk groups, each with a different cancer risk distribution, thereby facilitating adaptive cancer risk strategies. This method can enable the optimization of available resources and intervention timing based on particular biomarker sensitivity and specificity in predicting disease progression and prognosis among various risk groups that dynamically evolve over time. Of course the challenge that remains is arriving at study numbers adequately powered to achieve statistically interpretable outcomes.

Yet such an approach is not without precedent, albeit with considerably reduced levels of complexity: staging of cancers on account of histology has increased the granularity of our understanding and altered our approach to treatment. Progressively, this is further enriched as more and more clinically validated interventions and their associated biomarker solutions come online. Expanding on this principle by integrating clinical findings with research 'omics towards the construction of large scale Bayesian models (where the probability of given states is estimated based on a given set of starting points) promises a 'live' treatment and response scenario. Presently, this is done empirically. However, by continuously repositioning the collective understanding of such a diverse disease against the equally disparate patient background and treatment outcomes might indeed present a more realistic and accurate approach for contextual biomarker validation, understanding of disease mechanisms, [55,106] and personalized intervention to the benefit of all stakeholders: patients, clinicians and researchers.

To achieve this, it is necessary to work towards etymological and methodological concordance, or at the very least, provide adequate bridging principles, that will enable the necessary level of participation, i.e. on a global, continuous scale. However, there are few incentives to academics, institutions and the industry to share.

For example, transcriptomics is the study of the complete set of transcripts in a particular cell, tissue, sample or organism for a given physiological or pathological condition [107]. The transcriptome includes protein-coding messenger RNA (mRNA) and non-coding RNA (ncRNA: miRNA, long non-coding RNA (lnc-RNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and other ncRNAs) [108-110]. Since the transcriptome is a dynamic entity underpinning homeostasis, frequently altered during disease and treatment, transcriptome analysis has attracted a lot of attention in the study of EV function. This is challenged by the low abundance of EVs in biofluids. However, at least three approaches allow medium/high throughput detection of transcriptomic biomarkers including amplification based methods, hybridization-based microarrays, and Next Generation Sequencing (NGS). Amplification-based assays enable measurement of panels of both miRNA and mRNA and expand upon the concept of quantitative reverse transcription PCR (qRT-PCR). Popular examples include the SAbiosceinces PCR array, TaqMan OpenArray, TaqMan Gene Expression Assays, TaqMan TLDA microfluidic cards by Applied Biosystems, miScript miRNA PCR Array by Qiagen and miRCURY LNA qPCR by Exiqon. All these platforms offer high sensitivity and can precisely detect changes in tens to hundreds of individual nucleic acid levels whose existence is known *a priori* and for whom assays can be designed [111]. Advantages include a multi-log (5-9) linear dynamic range, resistance to purification protocol changes and organic contaminations. However, these technologies are mostly limited to medium throughput and annotated genes supporting hypothesis-derived

discovery [112]. Correlation and reproducibility of each platform in the context of EV-based biomarkers remains to be determined. It is, unfortunately, generally assumed that differences would reflect the minutiae of assay engineering differences with respect to target annotation and mechanism of amplification.

Hybridization-based assays (microarrays) are powerful tools for high-throughput evaluation of thousands of transcripts (hundreds to tens of thousands) in one assay and have been used in EV-based studies. There are two types of microarrays: short-oligomer microarrays (e.g. Agilent, Affymetrix Genechips, Nanostring), and long probe microarrays which include cDNA microarrays that may probe sequences up to a few hundred bases in length [113]. Microarrays are also limited to known target sequences, feature considerably reduced cost per analyte, but suffer less specificity, reduced dynamic range, and poor reproducibility [111]. Indeed, poor specificity has been demonstrated to drive discrepancies in gene-expression profiles between different probes targeting the same region of a given transcript [113,114], whereas operator and day-to-day variability are common problems in microarray data analysis [114]. For this reason, many a microarray-based study's outcomes are validated by qRT-PCR methods with little, if any, effort to detail and compensate for methodological bias or compatibility, but rather on the assumption that statistical significance across two analytical approaches is adequate.

Next generation sequencing combines the advantages of amplification-based 'omics with the throughput of microarrays to yield global sequence data agnostically [115,116] that can inform variability over single nucleotide polymorphisms (SNP) [117], alternative RNA splicing [118], copy number variations (CNV) [119], and differential expression (RNA-seq) [116] in a digital fashion. At first glance this is superior to either qRT-PCR or microarray approaches. Thus, the sequences of all transcripts in a sample are reverse transcribed into cDNA, prepared into a sequencing library,

read, bioinformatically mapped against a reference genome and individually quantified at a cost per analyte (base) orders of magnitude lower than microarrays. At ~US$400 several tens of terabytes of sequence data can be generated with billions of datapoints per sample. The resulting analytical feat requires expert computational know how and infrastructure to undertake. However, the technology is sensitive to the relative abundance of individual transcripts in a sample, amplification method artifacts, chemistry-related bias and detection technique-mediated error – notwithstanding computational limitations (aligner bias, reference genome bias). Thus, low frequency transcripts require 'deeper' (i.e. more) sequencing at a risk of artifact detection and mis-identification. Furthermore, as sequence ligation (adapters) is common in many sequencing library preparation methods, ligase sequence preference artifacts have been described to influence transcript frequency detection[120]. Moreover, nucleic acid contaminants arising from the biological origin of the processing enzymes can also contribute to confounding datasets [121,122]. Presently, each of the four most popular, commercially available RNA sequencing platforms has its own significant advantages and disadvantages. Briefly, Illumina NGS is an evolution of microarray technology and the most widely adopted platform, as it is less prone to error on account of homopolymer regions (e.g. adenosine multimers A_n, where $n > 6$). However, as with microarrays, it is based on imaging and base-by-base template extension, which makes it slow, costly and high maintenance. Moreover, it is subject to frequent chemistry and instrumentation updates that affect data quality and compatibility. On the other hand, it is the only approach that has been used to date to generate RNA-Seq data directly on histology sections by applying clonal template amplification on tissue sections [123]. The Ion Torrent technology is based on semiconductor microchip pH sensors of nucleoside addition. This detection methodology dramatically accelerates the data yield rates and enables robust hand-held NGS at

the bedside (DNA Electronics), but has a lower throughput than Illumina and is hampered by homopolymer errors and insertion/deletion artifacts. Both Illumina and Ion Torrent have <400 bp sequence limits and require sample fragmentation and clonal amplification; this causes problems in the analysis of repetitive regions and introduces further risk of error. Pacific Biosciences on the other hand uses optical, real time, single molecule sequencing which permits reads of up to hundreds of thousands of bases in length, but is a very slow, error prone, high cost and large footprint platform. Similarly, Oxford Nanopore also offers real time single molecule sequencing, this time on a sequencer the size of a USB stick that uses conductivity across a synthetic lipid bilayer to analyse transcript sequences as these transverse an engineered protein pore. It is considerably cheaper and faster than Pacific Biosciences, but it is a temperature- and kinetic energy-sensitive, considerably lower throughput instrument with a much higher error rate. In the next few years more robust, solid-state nanopore technologies based on graphene and other materials are expected to replace biological nanopores. Thus, where Illumina and Ion Torrent are good for counting non-repetitive sequences, Pacific Biosciences and Oxford Nanopore are better at developing complex repetitive sequence scaffolds and for RNA-SEQ splice variant enumeration. Few studies aim to bring together the advantages offered by each platform on answering clearly specific research questions. Rather the community is absorbed by the commercial marketing efforts and accessing answers with the least possible resistance, often with the least useful value. To the best of our knowledge these methodologies have not been evaluated yet in single EV sequencing and, by extension, to comparative sequencing of individual EVs. Notably, although for many years the cost of these technologies drove analysis to focus on single replicates, presently, independent biological and technical replicates are considered necessary [116].

In their effort to understand the role of EV in disease, the reader is directed to the several databases that have been introduced to publicly source datasets of studies investigating mRNA, miRNA, but also proteins in biofluids including Exocarta [124], ExcellmiRDB [124] and miRandola [125]. However, most of the studies in these databases utilized targeted as opposed to genome- wide association studies [126]. Since the targeted-approach is based on an *a priori* knowledge of gene function in disease pathogenesis, it is highly hypothesis-dependent and may overlook other active network components, negative/positive feedback loop elements and indeed RNA editing/splicing changes [127,128]. Moreover, false positive / negative error rates, in many cases not taken account of, confound data replication in follow-up studies [128]. The reader is therefore advised to approach the interpretation and extrapolation of these results with caution.

Eirin *et al*. [129], performed transcriptome profiling on EVs derived from mesenchymal stem cells of adipose origin. These contained mRNA for transcription factors (e.g. NRIP1, POU3F1) and genes involved in angiogenesis and adipogenesis, genes involved in TGFβ signaling pathway, and selected miRNAs. Gene ontology analysis revealed that these miRNAs might target genes and transcription factors that contribute to several cellular pathways, including angiogenesis, apoptosis, cellular transport, and proteolysis. Interestingly, this enrichment was selective; cytoskeleton and mitochondrial gene families were excluded from these EVs [129]. Contemporary to the explosion of interest in EVs, miRNAs became established as nodal regulators of gene networks: data indicated specific miRNA could drive cell phenotype. Thus many EV transcriptomic surveys focused on this class of RNA analytes exclusively to reveal that up to 76% of all mappable reads generated by RNA-Seq on EVs were indeed miRNA transcripts[11]. As was concluded from target gene enrichment analysis and functional experiments, these miRNAs might play important functions in protein phosphorylation, RNA splicing, and the modulation of

immune functions [3,11,47]. However, miRNAs are present in biofluids in three forms: cell-derived EVs, high-density lipoprotein particles, and Ago2 protein complexes [130,131]. The sorting of miRNA to the EVs is indeed specific and selective[3] and may include, amongst others, the miRNA motif and sumoylation of heterogeneous nuclear ribonucleoproteins (hnRNPs) C pathway [132], the neural sphingomyelinase 2 (nSMase2)-associated pathway [133], and the RNA induced silencing complex (RISC)-related pathway [134]. On the other hand, the mechanism of extra-vesicular miRNA release is poorly understood. The reader is thus advised to consider the impact of sample processing methodology on the outcomes reported between studies. Nevertheless, there is strong biomarker and clinical diagnostic potential in EV miRNAs [47,135-142]. **Table 6** demonstrates a summary of the use of EV-associated miRNAs in clinical settings for biomarker discovery and disease prognosis. Validation in large independent cohort studies, however, can sometimes be contradictory. For instance, differential regulation of a total of 143 miRNAs in plasma or serum in 32 breast cancer biomarker publications is demonstrated in which 100 deregulated miRNAs were reported in only 1 publication [54].

One possible explanation for these discrepancies suggests that miRNA expression changes in biofluids might occur earlier than conventional biomarkers, but this is often overlooked or not evaluated at all ahead of designing clinical validation studies. For example, in a cardiovascular ischemic events, circulating miRNAs (miR-1, -133a, and -133b) achieved their peak around 3h before the commonly used troponin I (TnI) peak [143]. Similarly, markers of inflammation and damage in cardiovascular disease, such as C-reactive protein and cytokines are observed in a later stage than miRNA deregulation Complexity also arises from the apparently non-specific elevation of certain circulating miRNAs such as miRNA-21 [135,145], which confound identification of disease-specific miRNA profiles. Integrating miRNA panel data in systems biology as

opposed to focusing on single miRNAs can, however, help facilitate patient classification. Alternatively, measuring commonly dysregulated miRNAs might offer value not in disease screening but in disease monitoring or prognosis studies [54]. For instance, the levels of three non-specific cancer-related miRNAs, miRNA-21, -221, and -141 in blood plasma of prostate cancer (PCa) patients have been demonstrated to be useful for predicting metastasis within patient subgroups [146]. Another challenge involves the data normalization approach used. Thus, in RNA studies, including miRNA, target levels are typically expressed relevant to at least one, but more commonly more than three different RNA targets not influenced by the disease/treatment, as determined through comprehensive profiling of normative samples for each biofluid. Indeed, many published studies provide selections of normalizing targets without adequate supporting evidence, perhaps beyond habitual use. These variables, in addition to sample processing, should be standardized ahead of attempting to establish clinical utility in independent cohorts [147]. Alternatively, a unified means of cross-study normalization, perhaps through automated selection of common normalization features (e.g. common normalizing gene subsets) could be adopted. Interestingly, since the number of EVs and their associated miRNAs is increased in various diseases, it has been proposed that using the same amount of starting material (μl of biofluid) might be a more suitable and adequate approach as compared to standardizing the amount of EV-associated miRNA extracts [24,47]. This is not dissimilar to the single analyte approach common to viraemia analytics used in research. However, the approach is superseded by the use of endogenous normalization and/or spike in controls. Thus, in the absence of a globally harmonized biomarker reporting and data integration system, it is our view that more meticulous studies, with better thought out controls, based on much larger patient cohorts along

with side-by-side comparisons with clinical parameters and conventional biomarkers are required for evaluating the utility of EV-associated miRNAs in the clinic.

EVs and DNA biomarkers

Genomic DNA biomarkers report genome-level changes using a variety of methods, including genome sequencing, qPCR and digital PCR [148] to accurately report SNPs, CNVs, genomic rearrangements and rare genetic sequences that functionally underpin the pathophysiology of disease [149]. This approach is most frequently used in oncology. However, tumor analytics have long been known to suffer operator and sampling biases. Thus, tumor heterogeneity is not fully represented within a given biopsy, irrespective of the analytical platform used. Yet genetic changes in tumor tissues are also mirrored in biofluids and EVs [20,150]. Importantly, these extracellular DNA sources may capture a snap shot of the disease state to be used for diagnosis, disease monitoring, and therapeutic stratifications – particularly in 'deep sampling' (i.e. NGS) or highly sensitive (e.g qPCR) methods [47], without the need for invasive biopsy. Presently NGS is restricted to highly specialized, centralised clinical settings worldwide with significant research activity and are not suited to disease screening in health care settings. NGS targeted to specific sequences relying on *a priori* data/hypothesis is more cost-effective, allows for deeper sampling of commonly mutated genes, can simplify NGS analytics and therefore is the leading approach pursued for diagnostic NGS dissemination [125]. Crucially, targeted NGS has comparable sensitivity and specificity for detection of disease specific mutations to Sanger sequencing, but benefits from significantly higher levels of data yield, can report unexpected mutations in key genes and inform digitally mutation abundance [125,151].

At first glance, the presence of DNA in EVs would indicate their apoptotic/necrotic nature (DNA fragments) as opposed to their enrichment in actively produced vesicles such as exosomes. As a result, the utility of EV-associated DNA has so far been less explored. Nonetheless, double strand DNA (dsDNA), mitochondrial DNA (mtDNA), single strand DNA, and oncogenic amplifications have reportedly been detected in EVs [103,152-156]. Double strand DNA was isolated from EVs originateing from different human cancer cell lines, including chronic myeloid leukemia and colorectal carcinoma [153]. Genomic DNA reflecting the mutational status of parental tumor cells was found in EVs [153,157,158]. DNA containing amplification of the oncogenic c-Myc was isolated from circulating EVs in glioblastoma patients [155]. In another study, >10kb fragments of double stranded genomic DNA were detected in exosomes originating from pancreatic cancer cells and sera of patients with pancreatic ductal adenocarcinoma. Mutations in KRAS and p53 were also detectable in the EVs isolated from patients' sera [159]. Additionally, whole genome sequencing demonstrated that serum exosomes from patients with pancreatic cancer contain genomic DNA originating from all 23 human chromosomes [159]. These findings suggest that EVs might hold value as translational biomarkers in identifying parental cell mutations. Elsewhere, mtDNA has been isolated from glioblastoma and astrocyte-derived EVs but their functionality remains unclear [154]. Moreover, Whole-exome sequencing and genome-wide copy number profiles of EVs isolated from plasma and pleural fluid showed robust representation of the tumor DNA within the shed EV compartment in patients with pancreaticobiliary cancers [150].

Crucially, actionable DNA mutations such as NOTCH1 (cell survival and apoptosis) and BRCA2 (DNA repair) as well as fusion genes with well-described causal roles in oncogenesis (e.g. APBA and STXBP1, ACOT1 and LMCD1) were found in circulating EVs [150]. Interestingly,

different subpopulations of EVs (i.e. exosomes, microvesicles) have also been reported to carry different amounts of gDNA [158]. For instance the relative ratio of PTEN, TP53 and MLH1 gDNA fragments was reported to vary by EV subpopulation as quantified by qPCR, using the GAPDH housekeeping gene as a reference [158]. It is unclear however if GAPDH is indeed an EV 'housekeeper', or a normalizer selected habitually from RNA studies. Nevertheless, collectively, these studies suggest that the DNA content in circulating EVs might not reflect parent cell viability, but perhaps active shedding of genomic fragments as they become compromised through genomic instability, and lend further support to their clinical evaluation as minimally invasive liquid biopsies. However, more high-throughput studies are needed to establish the functional significance of EV- associated genetic material in various disease [156].

Systems medicine approach and EV-associated biomarkers

To date, the amount of validated biomarkers is considerably disproportionate to biomarker discovery investment [160-162]. A large fraction of these funds were dedicated to studies on specific biomarkers of interest at selected time-points, as opposed to the agnostic discovery followed by kinetics description and hypothesis-driven validation model of biomarker development and clinical translation. Emerging during the 'omics era, EV biomarker studies have fared better and have benefited from inherent advantages that enhance their utility in disease detection, stratification, prognosis and monitoring in the context of personalized medicine [47,140,163-165]. Furthermore, multi-analyte biomarker studies have enabled integrative analytical approaches to data mining [166], which cannot be implemented in single biomarker studies in accounting for complex phenotypes and stochastic alterations. From a biological stand point, single molecule variability within and across diseased and healthy individuals is subject to inherent biological noise not statistically accounted for in focused studies [167]. The conceptual framework of

integrating 'omics data, systems biology approaches, and personalized medicine in EV biomarker studies is depicted in **Figure 4**. Thus, multiple studies can be used to identify biomarker sets (signatures) instead of single biomolecules, in a temporally robust manner not subject to irrelevant changes such as circadian rhythms (Table 5). Integration of affected biomarkers in systems biology models can inform the affected disease pathways, leading to the identification of causal biomarkers instead of simply correlational outputs and, by extension, point the way to nodal points of pharmacological intervention in a quantitative fashion [168]. Most importantly, the system approach takes into account the interrelating biological roles of pathway components, making it less sensitive to biological heterogeneity.

Eventually, the concepts of stratified medicine and systems biology are expected to drive personalized medicine into producing truly tailored treatments based on underlying disease mechanisms relevant to individual patients. However, to achieve this, a range of novel disease-specific biomarkers with relation to specific dysregulated pathways needs to be identified. In addition, a reference profile for cell-specific and tissue specific EVs molecular signatures is needed. Qualitative and quantitative modeling of EV molecular signatures can pave the way for EV-based monitoring and prospective diagnosis.

Conclusions

EVs continue to gain increasing attention as major players of cell communication with strong potential as causal, clinical biomarkers. Translational success will pivot on appropriate quality assurance and method validation across the continuum of discovery to clinical implementation. As stable reservoirs of different biomolecules, EVs suffer fewer challenges than other analyte

matrices, and have the potential to serve as high value liquid biopsies in clinical diagnostics. Profiling of EVs can accommodate tumor heterogeneity and can be relatively to completely non-invasive, based on the biofluid selected. The substantial progress in the isolation, characterization, and elucidation of the biogenesis and functional roles of EVs in various physiological and pathological states is balanced by the major challenges and urgent need for methodological harmonization and better study structure. To translate EV utility from discovery to the clinical setting these challenges must be met at the pre-analytical, analytical and post-analytical phases. In this context, the adoption of systems biology approaches is likely to help resolve the analytical challenge of 'omic datasets, enabling focus on causal biomarkers and the transition of EV-based diagnostics to the exciting opportunity of truly personalized medicine.

Table 1- Extracellular vesicles (EVs) isolated from different cell types

Cell type	Class	Tissue origin	EV type	Cargo of EVs	Biological function	Reference
B cells	Primary	Haematopoietic	Exosomes	B220 (CD45R), BCR complex, CD9 and CD81 tetraspanin MHC-I and MHC-II	Interaction with extracellular matrix	[169,170]
Huh 7.5 cells	Immortalized	Hepatocarcinoma cell line	Exosomes	miRNA-122	Sensitize monocytes to LPS and ethanol effect and induce pro inflammatory phenotypes in monocytes	[3]
THP1 cells	Immortalized	Human acute monocytic leukemia	EVs	miRNA-27a	miRNA-27a cargo in monocyte-derived EVs can polarize monocytes into M2 macrophages.	[98]
Human T cell blasts	Primary	Haematopoietic	Microvesicles	bioactive Fas ligand and APO2 ligand	Promoting activation induced cell death	[171,172]
TS/A cell line	Immortalized	Mammary adenocarcinoma	Exosomes	PGE2, TGF-β	Suppress immune responses, modifying myeloid precursors toward a more tolerogenic phenotype	[173]
MML-1 cells	Immortalized	Melanoma	EVs	miR-214-3p, hsa-miR-199a-3p and hsa-miR-155-5p	Melanoma progression	[174]
Renal cancer stem cells	Immortalized	Human Renal Cancer	Microvesicles	proangiogenic mRNAs and microRNAs	Stimulate angiogenesis, formation of lung pre-metastatic niche	[5]

Table 2- Disease-related EVs isolated from different human biofluids.

Type of biofluids	Disease	Type of EVs	Molecular Cargo	Changes in number of EVs in the disease state	Reference
Serum					
	Alcoholic hepatitis	Exosomes	miRNA-122	Increased	3
	Glioblastoma	Macrovesicles	EGFRIII fusion	Increased	[152]
Plasma					
	Melanoma	Exosomes	High protein content including Met oncoprotein, CD44, Hsp70	No change	[175]
	Nasopharyngeal carcinoma	Exosomes	EBV BART viral miRNA	Unknown	[176]
	Hepatitis C	Exosomes	Hepatitis C virus, miRNA-122	Increased	23
Milk					
	Staphylococcus aureus infection	Exosomes	bta-miRNA-142-5p, miRNA-223	Unknown	[177]
Cerebrospinal fluid (CSF)					
	Alzheimer's disease	Exosomes	miRNA-9, miRNA-125b, miRNA-146a, miRNA-155	Unknown	[178]
	Glioblastoma	Macrovesicles	CD144, CD4, CD45	Increased	[179]
Saliva					
	Healthy donors	Exosomes	Different miRNAs	Not applicable	[180]
Pleural effusion					
	Pancreaticobiliary cancers	Exosomes	Genomic DNA and transcriptome reflecting copy number profiles, point mutations, gene fusions and mutational signatures	Unknown	[150]
Urine					
	Incipient Diabetic Nephropathy	Exosomes	miRNA-145, miRNA-155, miRNA-130a	No change	[181]
Amniotic fluid					
	Mid-trimester of healthy pregnant women	Exosomes	Tubulin, Hsp72 Hsc73	Not applicable	[71]

Table 3: Role of extracellular vesicles in pathogenesis of different diseases

Disease	Type of vesicles	Reference
Infectious disease		
Parasitic trematodes/nematodes: immunomodulation	Exosomes	[182]
Spongiform encephalopathies: spread of transmissible prions via the blood	Exosomes	[183]
HIV: miRNAs transport involved in HIV-associated neuronal dysfunction, trans-infection of CD4+ T-cells	Exosomes	[184, 185]
HCV: shuttling virus between hepatocytes, transfer of viral replication components	Exosomes	23, [186]
Epstein–Barr virus: viral biogenesis and egress, exosome-dependent immune suppression in EBV-associated lymphomas	Exosomes	[187, 188]
Cancer		
Promote angiogenesis, thrombosis, and tumor cell proliferation	Exosomes, Microvesicles	[189-192]
Promote a pro-tumor environment to harbor metastatic niches and formation of pre-metastatic niche in different organs	Exosomes, Microvesicles	[193, 194]
Modulating bone marrow-derived cells to generate a pro-vascular phenotype	Exosomes	[175]
Induce immune suppression favoring tumor escape mechanisms	Exosomes, Microvesicles	[195-197]
Liver disease		
Cross-talk and horizontal transfer of miRNA between hepatocytes and monocytes	Exosomes, Extracellular vesicles	3, [98]
Mediate intercellular communication between hepatocellular carcinoma cells	Extracellular vesicles	[198]
Neurodegenerative disease		
Parkinson disease: Transfer of αsyn and inducing autophagy	Exosomes	[199]
Regenerative and protective functions		
Human mesenchymal stem cell-derived EVs protected against glycerol or cisplatin-induced kidney injury	Extracellular vesicles	[200,201]

Table 4: Overview of extracellular vesicle isolation techniques

Isolation Method (basic principal)	Indication	Advantages	Disadvantages
Ultracentrifugation (sedimentation based on size and density)	Large volume of biofluids	Most widely used standardized method, Can be combined with size exclusion and sucrose gradient method [21]	Low efficiency, Long protocol, Costly, Recovery dependent on rotor k factor and viscosity and sedimentation efficiency [21,74], risk of contamination/co precipitation with viruses [23], PA* [21], and LPP∓ [202]
Size exclusion (filtration+ chromatography)	Large volume of biofluids, Can be combined with Nano-membrane ultrafiltration concentrators [9]	Feasible, Inexpensive, Non-selectivity ϕ	Does not concentrate the EVs, Forcing EVs through filters may cause deformation and breakup of large vesicles [24]
Immune affinity isolation (antibody against specific EVs surface proteins**)**	High purity isolation of EVs, Isolation of sub-set of EVs, Isolation of EVs from viruses and LPP∓	High specificity and selectivity [203], Isolating special sub-set of EVs and negative selection [21,23], Easy to be coupled with beads and low speed centrifugation	Cross reactivity of antibody, Costly, Low yield [78], Expensive equipment
Microfluidic techniques (trapping EVs in micro channels)	Low volume of input biofluids	Can be combined by immune affinity methods [204]	Early stage of development, Low throughput, Lack of evidence regarding efficiency and downstream clinical utility in comparative studies, channel blocking.
Polymeric precipitation methods (Reduce EV solubility and drive precipitation by dissolving polymers)	Both low and high volume of input biofluids	Technically not sensitive, Efficient isolation, High yield of EV recovery and EV-associated RNA [9], Efficiency in clinical studies, Can be combined by immune affinity methods to increase purity [9,23]	Cannot appreciably purify EVs from a protein mixture and viruses unless coupled with immune affinity methods[23]
Sieving methods (Deriving filtration by pressure or electrophoresis) [205]	Very low amount of input material (3ul-4ul) and rapid isolation	Shorter separation time compared to size exclusion [205]	Low exosome recovery, Not suitable for large volume of biofluid, Lack of comparative studies and validation on clinical samples [204]
Porous structures (Capturing EVs through porous microstructures based on ciliated micropillar structure) [206]	Selectively trap particles in the range of 40-100 nm based on the research question	Fast trapping	Not suitable for isolation of larger particles, Not validated with clinical samples, Not suitable for handling large volume of biofluids, No analysis of cargo or comparative study available [204], Time consuming to characterize the EVs isolated based on this method ɣ [204 206]

*PA: protein aggregates; ∓ LPP: lipoprotein particles; ϕ Non-selectivity can be advantage in case that the researchers/clinicians are interested in isolating whole population of EVs and can be a disadvantage when isolation of a subset of EVs is of interest; ɣ to proceed with imaging and characterization, it is necessary to dissolve the silicon nanowire in PBS buffer overnight.

Table 5: Factors that affect the biomarker concordance during the course of the disease

Causal factors	*Examples of the effect of the causal factor in EV-biomarker concordance*	*References*
Clinical and biological		
Circadian exosomal marker expression	Circadian exosomal expression of renal thiazide NaCl cotransporter and prostasin in urinary exosomes	[207]
Change of exosomal cargo in different course of disease	Changes in the miRNA-cargo content within EVs as a mechanism influencing bone metastatic colonization	[100]
Heterogeneity in tumor microenvironment and tumorigenesis mechanisms	40% of all breast cancers contain hypoxic microenvironments that produce EVs with specific signature (contains miRNA-210)	[46, 208]
Choosing the most clinically relevant biomarker	mRNA transcript levels and corresponding protein showed marked differences in side by side measurements	[209]
Individual variability (age, gender, genetic factors, ethnicity)	Both qualitatively and quantitatively, the protein content of EVs showed gender specificity in renal tubule-specific responses in infected rats; EVs isolated from prostate cancer tumors showed ethnically and tumor-specific signatures	[91, 93, 210]
Diet factors	EVs derived from palmitate-treated cells were enriched in palmitate and transferred the deleterious effect of palm oil to muscle cells	[95]
Physical activity	Physical exercise induces rapid release of exosome subpopulation of extracellular vesicles into the circulation	[96]
Variation in signature of different subpopulation of EVs	Deep sequencing data showed the LIM1863 cells release different subpopulation of EVs harboring specific miRNA signature	[99]
Difference between animal models and human studies	Differential expression level of miRNA-122 in alcoholic hepatitis mouse model and human subjects with alcoholic hepatitis	[47]
Technological, analytical and sample handling factors		
Difference in detection frequency of different techniques	Differences in detection frequency of IDH1 mutation copy number in CSF of patients with Galioblastoma by BEAMing and Droplet Digital PCR Analysis	[67]
Increasing time between venipuncture and centrifugation	Induce rapture of EVs	[211]
Limitation in type of biomarkers	miRNA biomarkers and protein biomarkers showed reduced sensitivity compared to combination of miRNA/proteins for pancreatic cancer diagnostics	[61]
Collection, storage, and preservation of EVs	Freezing at -20°C caused a major loss in urinary EVs in contrast to storing at -80°C which lead to complete recovery of EVs compared to fresh urine, vortexing after thawing increase exosome recovery	[212]
High throughput sensitivity, dynamic range, and cost effectiveness	RNA-seq provide broader dynamic range compared to microarray	[213]
Choice of anticoagulant for plasma samples	Heparin can cause false negative PCR reads	[214]
Challenges in accurate measurement of cargo	Agilent Bioanalyzer small RNA kit is less accurate in quantifying miRNA after isolation of EVs, since presence ribosomal RNAs are not consistent in EVs	[88, 147]

Table 6: Examples of EV-associated miRNA dysregulation detected in human disease

Differentially expressed miRNAs	Disease	Biofluid	Controls	Type of EVs	Isolation methods	Spike-in/endogenous controls	Ref	Independent confirmation study
miR-21	Increased in Hepatocellular Carcinoma	Serum	Healthy controls/Chronic hepatitis B	Exosomes	Total Exosome Isolation Reagent (Invitrogen)	U6 snRNA	[135]	None
miR-192, miR-30a, miR-122	Increased in alcoholic hepatitis	Plasma	Healthy controls	EVs	Filtration/ ExoQuick (System Biosciences)	miR-15a, spiked in Cel-39	[47]	miR-122[3]
A multi-biomarker panel (RNU6-1/miR-16-5p, miR-25-3p/miR-320a, let-7e-5p/miR-15b-5p, miR-30a-5p/miR-324-5p, miR-17-5p/miR-194-5p)	Increased in locally advanced esophageal adeno-carcinoma	Serum	Healthy controls/ Barrett's esophagus	Exosomes	ExoQuick	Global normalization	[136]	None
miR-126, miR-199a	Increased levels inversely predict cardiovascular events	Plasma	Patients with stable coronary artery disease.	Micro-vesicles	Ultracentrifugation	spiked in Cel-miR-39	[137]	None
miR-375, miR-141p	Increased in prostate cancer	Urine	Healthy controls	Exosomes, Micro-vesicles	ExoMir extraction	snoRNAs (RNU44 and RNU48), Cel-miR-39	[138]	miR-375[140]
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a	Increased in colon cancer	Serum	Healthy control	Exosomes	Ultracentrifugation	Global normalization , miR-451	[59]	None
let-7f, miR-20b, miR-30e-3p	Decreased in non-small cell lung cancer	Plasma	Healthy controls	Micro-vesicles	Immuno magnetic beads	miR-142-3p and miR-30b	[139]	None
miR-1290, miR-375	Higher levels associated with poor survival	Plasma	castration-resistant prostate cancer patients	Exosomes	ExoQuick	miR-30a-5p, miR-30e-5p	[140]	None
miR-29c	Negatively associated with early renal fibrosis in lupus nephritis	Urine	Healthy controls/ non-lupus chronic kidney disease	Exosomes	Ultracentrifugation	GAPDH (mRNA), RNU6	[141]	None

Box1- Classification of extracellular vesicles

Historically, EV classification was based on cellular origin. However, EVs can be more accurately categorized on the basis of their biogenesis.

Oncosomes: tumor microvesicles that transmit signaling complex between cells.

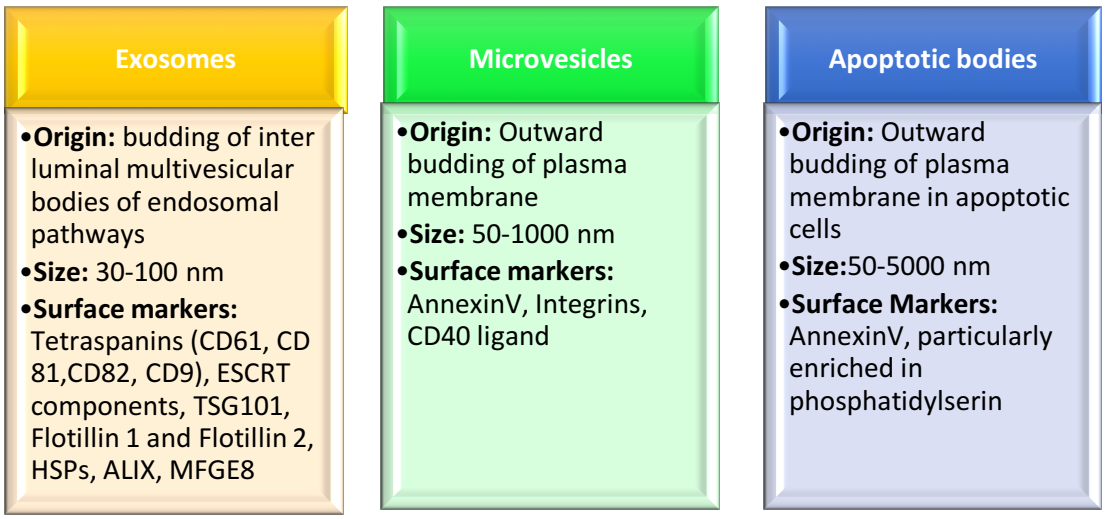
Ectosomes: vesicles secreted by human polymorphonuclear leukocytes

Microparticles: vesicles originated from pellets

Dexosomes: vesicles released from dentritic cells

Texosomes: vesicles derived from tumor cells

EV classification based on mode of biogenesis



*ESCRT: Endosomal sorting complex required for transport complex, MFGE8: milk fat globule-EGF factor 8 protein, TSG101: tumor susceptibility gene 101, HSPs: heat shock proteins

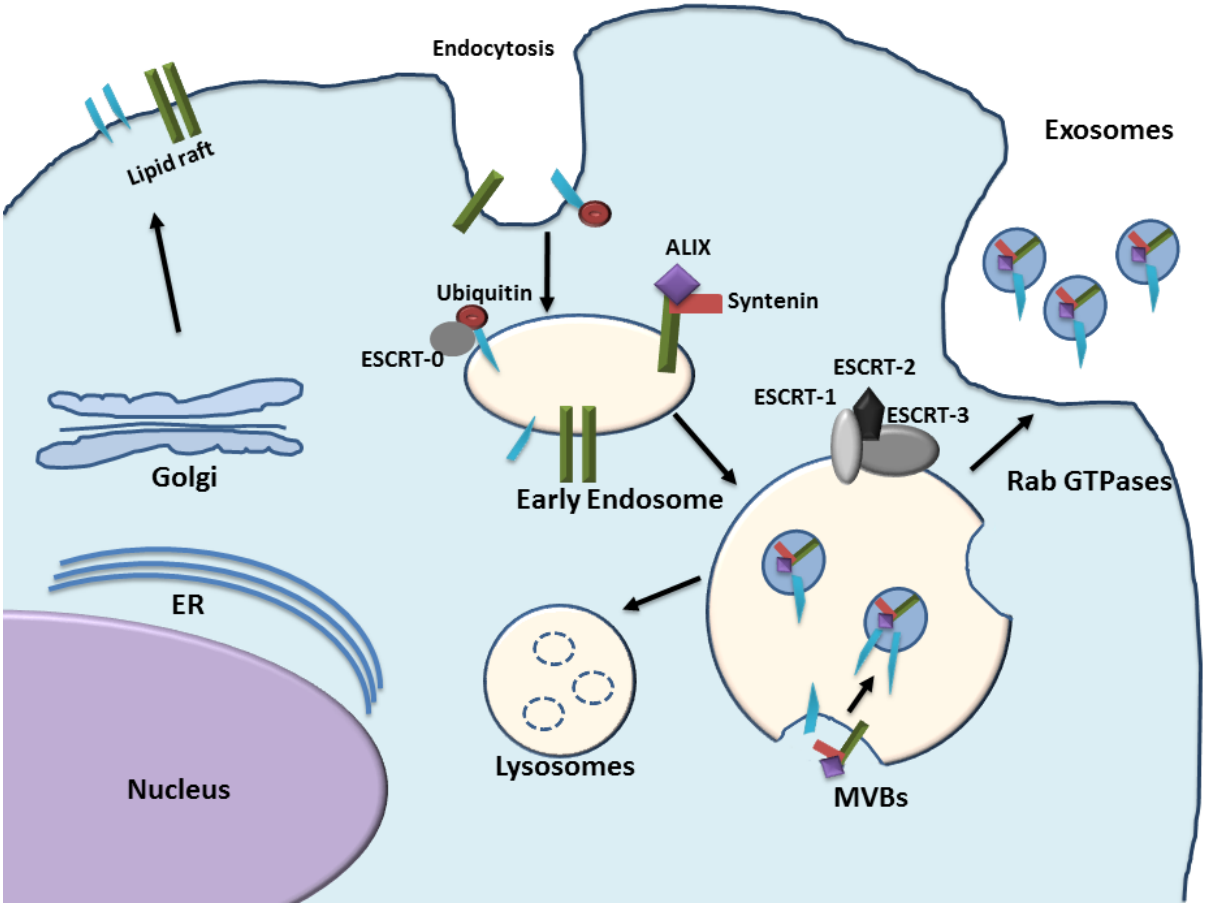


Figure 1- Exosome biogenesis and secretion- Exosomes are generated from multivesicular bodies (MVBs) of the endosomal system. Early endosomes formed after endocytosis and from MVBs in which cargo is packed in the exosomes by inward budding of the membrane. ESCRT machinery, mono-ubiquitination and the lipid raft and segregation into microdomains by ceramide has been described as facilitators to the exosome biogenesis. MVBs can merge with lysosomes resulting in degradation of the cargo or MVBs can merge with the plasma membrane which results in exosome release. This process is regulated by a by Rab GTPases. Exosomes contain different cargoes including Rab proteins, ALIX, MHC molecules, clatherins and transferrin receptors.

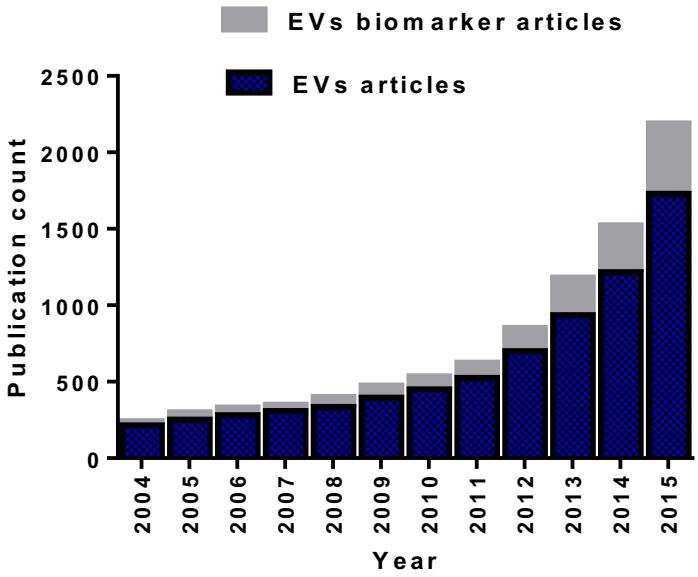


Figure 2- Volume of EVs publications. The bar chart shows the number of publications in general topic of EVs and related to the search term "extracellular vesicles OR exosomes OR microvesicles AND biomarkers" that were listed on PubMed between January 1, 2004 and December 31, 2015.

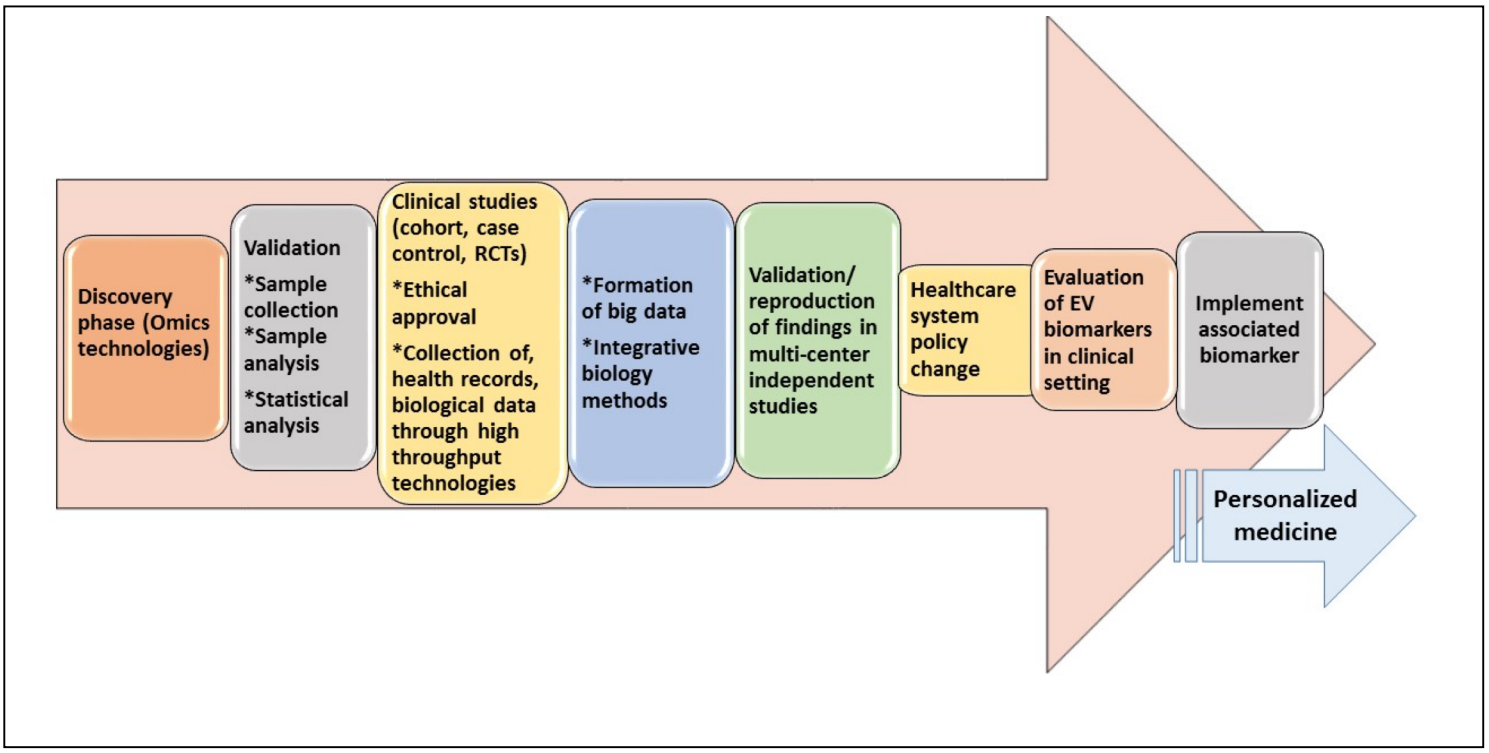


Figure 3- The EV biomarker workflow. An illustration of a pipeline in taking an EV-associated biomarker from bench to bedside

Figure 4- Frame work of integrating omics data and system biology approach in EV biomarker studies

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