Exosomes in bodily fluids are a highly stable resource of disease biomarkers

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Abstract

Biomarkers are measurable indicators of a biological state. As our understanding of diseases meliorates, it is generally accepted that early diagnosis renders the best chance to cure a disease. In the context of proteomics, the discovery phase of identifying bonafide biomarkers and the ensuing validation phase involving large cohort of patient samples are impeded by the complexity of bodily fluid samples. High abundant proteins found in blood plasma make it difficult for the detection of low abundant proteins that may be potential biomarkers. Extracellular vesicles (EVs) have reignited interest in the field of biomarker discovery. EVs contain a tissue-type signature wherein a rich cargo of proteins and RNA are selectively packaged. In addition, as EVs are membranous structures, the luminal contents are protected from degradation by extracellular proteases and are highly stable in storage conditions. Interestingly, an appealing feature of EV-based biomarker analysis is the significant reduction in the sample complexity compared to whole bodily fluids. With these prescribed attributes, which are the rate-limiting factors of traditional biomarker analysis, there is immense potential for the use of EVs for biomarker detection in clinical settings. This review will discuss the current issues with biomarker analysis and the potential use of EVs as reservoirs of disease biomarkers.

Keywords

exosomes; extracellular vesicles; microvesicles; biomarkers; bodily fluids

Introduction

Biomarkers are tissues and/or bodily fluid-based measurable indicators of a biological condition [1][2]. Biomarkers include DNA, RNA, proteins and metabolites that can reflect an individual's state of health or disease [3, 4]. The National Institutes of Health Biomarkers Definitions Working Group in 1998 defined a 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" [5]. Based

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on their utility in practice, biomarkers can provide insights on diagnosis, prognosis, regression or response to treatment of a disease [6, 7]. Currently, invasive and non-invasive procedures are often employed to identify biomarkers [8]. In the context of cancer, tumour tissues are obtained by invasive biopsies for biomarker analysis. In cases where tumours or diseased tissue (e.g., neurodegenerative diseases) are inaccessible, non-invasive methodologies are relied upon [9]. Non-invasive methods of analysis include the detection of the biomarker(s) in bodily fluids. One of the attractive features of using bodily fluids is that it also allows for the early diagnosis of the disease [8]. A variety of bodily fluid samples including serum, plasma, urine, cerebrospinal fluid, saliva, ascites and amniotic fluid have been commonly analysed for both identification and validation of potential biomarkers [3].

Current state of biomarker analysis

The idea of monitoring serum proteins from blood samples is well established in terms of monitoring tumour growth: for instance, the detection of prostate specific antigen (PSA) for prostate cancer, carbohydrate antigen 125 (CA125) for ovarian cancer and carcinoembryonic antigen (CEA) for multiple cancers [9]. Unfortunately, this approach is not always plausible due to the paucity of tumor specific biomarkers. Compared to other bodily fluids, plasma or serum samples are considered ideal for mining biomarkers for clinical practice as blood contains a sampling of every tissue in the body and can be considered as a universal source of biomarkers [10, 11]. However, proteomic strategies have proven to be difficult in plasma mainly due to the vast dynamic range of proteins contained within plasma [10–12]. With 99% of plasma protein mass made up with 22 abundant proteins, it remains difficult to detect other low abundant proteins [10]. Abundant plasma proteins such as albumin, fibrinogen, immunoglobulins, transferrin, lipoproteins and plasminogen alone account for >90% of the total protein content in blood. Several methods based on immunoaffinity and columns have been developed to deplete the high abundant plasma proteins including albumin and immunoglobulins [13–16]. Even though the methods can successfully deplete some of the abundant proteins, plasma still remains a complex fluid encompassing proteins from many normal tissues and cell types. Additionally, the immunodepletion methods suffer from nonspecific binding, increases the variability between samples and thus sacrifices biomarker screening efficiency [10, 11, 17].

To circumvent the complexity of analysing bodily fluids, the discovery phase is usually carried out in less complex samples such as panel of cell lines. The ensuing validation of potential biomarkers is performed by targeted analysis in bodily fluid samples or in tissue sections [8]. To quantitatively measure the potential biomarker of interest in control and disease samples, immunoassays are employed, ELISA being the gold standard [10]. These current approaches are constrained with the limit of signal detection for clinical biomarkers and the lack of well characterized highly specific monoclonal antibodies [18]. With all the issues described in identifying biomarkers in the discovery phase by proteomic strategies, extracellular vesicles can potentially overcome these challenges and have reignited interest in biomarkers. This review will focus on the utility of extracellular vesicle fractions from bodily fluids for biomarker analysis.

Extracellular vesicles

Extracellular vesicles (EVs) are secreted by various cells into the microenvironment. Based on the biogenesis, EVs can be classified into three categories; apoptotic blebs, shedding microvesicles (SMVs) or ectosomes and exosomes [19, 20]. SMVs (100–1000 nm in diameter) occur through the budding of the plasma membrane [21–24]. Under pro-apoptotic stimuli, a cell undergoes apoptosis and subsequently releases apoptotic blebs [25–27]. Exosomes are membranous nanovesicles with a diameter range of 40–150 nm and float in sucrose gradients at a density range from 1.13 to 1.19 g/mL [19, 28]. Even though multiple attributes of exosomes are poorly understood, exosomes remain the most studied among the EVs [29]. The nomenclature of EVs is yet to be standardized and thus allowed for confusion in vesicle naming [20]. In addition, no vesicle type could be isolated to homogeneity due to the unavailability of molecular markers that differentiate the classes of EVs [30]. For this reason, most of the EV-based studies have isolated a mixed population of vesicles irrespective of the sample source (bodily fluids or cell culture media). However, in the context of biomarkers, the inability of purifying vesicle types to homogeneity may not be a concern as far as patient samples can be differentiated from healthy volunteers.

Exosomes emerge from the endocytic pathway. Invagination of the plasma membrane results in early endosomes that matures into late endosomes or multivesicular bodies (MVBs). Inward budding of the limiting late endosomal membrane forms the intraluminal vesicles (ILVs) [31, 32]. The ILV's are released into the extracellular space by the fusion of MVBs with the plasma membrane and are called as exosomes thereafter [19, 31, 33]. Exosomes have been reported to be secreted by a wide range of cells including immune, neuronal and cancer cells [34–38]. Similar to cells, exosomes have a lipid bilayer membrane consisting of a range of lipids and proteins [39] as well as luminal proteins and RNA [19]. The molecular content of exosomes and the biological function that they perform are influenced by the cell type of origin [40]. For example, exosomes released by dendritic cells contain co-stimulatory proteins necessary for T-cell activation while most exosomes derived from tumour cells do not [41]. Similarly, platelet-derived exosomes promote tumour progression and metastasis of lung cancer cells [42] while ovarian cancer exosomes stimulate angiogenesis [43]. Most importantly, exosomes are shown to contain oncoproteins which can be transferred to target cells upon fusion/uptake [44]. Exosomes are shown to regulate many biological functions including, immune response regulation [45, 46], antigen presentation [47, 48], the transfer of RNA and proteins [49, 50], transfer of infectious cargo [35, 51, 52], non-classical secretion of proteins [53] and intercellular communication [54–57]. Also, exosomes have been involved in the progression of disease by the transfer of oncogenes in cancer [56] and pathogenic proteins between neurons in neurodegeneration [58–60]. The release of exosomes into the extracellular space affords an opportunity to examine exosomes in body fluids such as blood, urine and malignant ascites [61]. Accessing these bioactive vesicles in a non-invasive manner may lead to potential biomarkers for diagnostic and prognostic purposes.

For the purpose of this review, all the vesicles will be referred to as EVs from here on.

Advantages of EVs in biomarker analysis

As EVs circulating in blood are likely to have been derived from various tissues throughout the body, the isolation of cell specific EVs can provide us with information on a specific pathological condition [19, 62]. Bodily fluids contain EVs and creates unparalleled opportunities to exploit these vesicles as reservoirs of disease biomarkers [62, 63]. Disease derived EVs can relay information which can be used to determine the state or progress of disease as well as optimal modes of treatment [64]. Importantly, exosome based biomarker analysis have many significant advantages compared to conventional strategies which are discussed further (Table 1).

EVs contain disease causing proteins

As EVs may contain disease causing proteins including mutant proteins/RNA, assaying for mutant or disease causing proteins/RNA as disease biomarkers may provide the required specificity for a biomarker test [8]. Wang *et al.* used MS-based selected reaction monitoring (SRM) and were able to distinguish between wild type and mutant forms of the KRAS protein (G12D) in cell lines, tissues samples and bodily fluids [65]. At least in this context, the study showed that the SRM technique could easily be used on complex biological samples with high sensitivity (~10 fmol). Mathivanan *et al.* identified 57 mutated proteins from the secretome of 18 cell lines representing different stages and underlying mutation status of colorectal cancer using MS [66]. The possibility of an altered extracellular localization of a mutated protein allows biomedical researchers to exploit such mutant proteins as cancer biomarkers. As wild type proteins can also be expressed in multiple tissues, using them as candidate biomarkers of a disease often lacks the specificity. Currently, CEA is the most widely used biomarker associated with colorectal cancer screening. However, the lack of sensitivity and specificity of the test renders it unsuitable for clinical screening. Elevated serum levels of CEA are not only detected in colorectal cancer patients but also in lung, cervix [67], breast [68], gastric [69] and pancreatic [70] cancer patients. The use of mutant proteins that are also drivers of the disease may provide the much needed specificity that seems to lack from wild type proteins like CEA. Similarly, mRNA of the fusion gene TMPRSS2:ERG was detected in EVs isolated from the urine of prostate cancer patients [71]. In addition, oncogenic receptor EGFRvIII is shown to be released by EVs [41, 44, 72]. Based on these observations, MS techniques including SRM can be utilized for the identification of disease causing proteins in EVs.

EVs are reservoirs of disease biomarkers

EV-based biomarker analyses are carried out with the ultimate aim to identify biomarkers for early diagnosis and prognosis of disease conditions [73, 74] (Fig. 1). Recently, the use of EVs as indicators for response to treatment has also gained significant interest [75, 76]. Ideally, EV-based protein signatures could predict the outcome of a treatment thereby allowing for strategizing treatment options as well as reducing significant costs associated with the treatment that is bound to fail.

Currently, the diagnosis and surveillance of prostate cancer utilizes PSA [77, 78]. However, PSA lacks specificity for prostate cancer and hence has the risk of over diagnosis and

overtreatment [77, 78]. Nilsson and colleagues identified two distinct potential mRNA biomarkers, PCA-3 and TMPRSS2, in prostate cancer EVs which can be utilised in prostate cancer diagnosis [64, 71]. Similarly, Duijvesz and colleagues observed the proteomic profile of EVs from four prostate cancer epithelial cell lines PNT2C2, RWPE-1, PC346C and VCaP [77]. Following tryptic digestion and LC-MS/MS analysis, 1494 non-redundant peptides were identified. The authors have validated PDCD6IP, FASN, XPO1 and ENO1 by Western blotting and immunohistochemistry. While PDCD6IP and ENO1 are most often identified in EVs (ExoCarta [79] and Vesiclepedia [80]) and may lack the specificity, the other two proteins could be potential biomarkers. However, independent validation of the candidate markers is still needed on large patient cohorts.

It has been previously reported that EGFRvIII containing EVs could be detected in the serum of glioblastoma patients [41]. Shao and colleagues utilised a rapid and highly sensitive technique for the isolation and protein typing of EVs from glioblastoma patient blood samples [75]. EVs were labelled with target specific magnetic nanoparticles and introduced into a microfluidic chip and detected via miniaturized nuclear magnetic resonance system (μNMR). This system was able to differentiate glioblastoma EVs from non-tumour host derived EVs and predict treatment response. Reflective of the protein profiles of the parental cells, glioblastoma derived EVs exhibited elevated levels of EGFR, EGFRvIII, PDPN and IDH1 R132H compared to control EVs [75]. Hence, the detection of tumour derived EV signature via a blood test has the potential to provide diagnostic, prognostic and survival value [41, 56, 75, 81].

Peinado and colleagues isolated EVs from blood plasma of patients with different clinical stages of melanoma [56]. While the quantity of EVs was not affected significantly, the protein content within EVs increased in advanced melanoma patients. Additionally, patients with low protein content within EVs exhibited an increased survival advantage over patients with protein rich EVs. Following tryptic digestion and MS analysis, melanoma cell-derived EV protein signature (human and mice) was identified. Compared to the control EVs, TYRP2, VLA-4 and HSP70 were highly abundant in advanced melanoma cell-derived EVs. Interestingly, an isoform of HSP70 was identified in advanced melanoma cell-derived EVs which has been reported to be involved in cellular transformation [56, 82].

EpCAM-based immunoaffinity pull down of EVs derived from colorectal cancer patient plasma revealed a statistically significant amount of EpCAM-positive EVs as compared to healthy controls [83]. These findings were consistent with Taylor and colleagues in the context of ovarian cancer where the levels of EpCAM-positive EVs correlated with clinical stages of patients [84]. In addition, circulating EVs derived from cancer patients were correlated with disease prognosis and survival [83]. Consistent with previous studies, there were no observed differences in disease free survival between patients with high and low levels of circulating EVs. However, patients with high levels of circulating EVs were shown to have a shorter overall survival compared to patients will low EV levels. After 36 months of follow up, the overall survival of patients with high and low levels of EVs was 55 and 89%, respectively [83].

The possibility of exploiting urinary EVs as source of disease biomarkers beyond the urinary tract was investigated by Li and colleagues [85]. The proteome of urinary EVs from both healthy and non-small cell lung cancer (NSCLC) patients were examined. Urinary EVs isolated by ultracentrifugation were separated by 1-D SDS-PAGE, subjected to tryptic digestion followed by nano-HPLC-chip-MS/MS. Among the 18 proteins identified, leucine-rich α-2-glycoprotein (LRG1) was chosen to be investigated further and Western blotting confirmed the presence of LRG1 in association with EVs in both subjects with approximately a 6-fold increase in NSCLC patient urinary EVs. Additionally, immunohistochemistry was performed on NSCLC tissue sections showing high LRG1 expression levels compared to adjacent non tumour lung tissue. A combination of these findings implies the use of urinary EVs as biomarkers and a non-invasive tool for the clinic [85, 86].

EVs isolated from bodily fluids are less complex than whole bodily fluids

The complexity of bodily fluids is a rate limiting step in the identification and validation of biomarkers in conventional analysis. When the EV fraction of any bodily fluid is targeted, the complexity of the sample is significantly reduced (Fig. 2). For instance, Kalra *et al.* isolated EVs from plasma and established the depletion of high abundant plasma proteins [63]. Antibody-based high abundant protein depletion strategies may create variability between patient samples by non-specifically depleting low abundant proteins. Whilst different EV isolation methods may yield varying results [87, 88], it can be speculated that the same centrifugation based method within a set of patient samples seldom create any variability.

Membranous EVs are highly stable

Biorepositories store patient samples for decades. The stability of EVs and their luminal cargo in stored patient samples is critical for biomarker analysis. Sokolova and colleagues assessed the stability of EVs isolated from three different cell lines (HEK293T, ECFC and MSC) in PBS at 37°C, 4°C and −20°C [89]. Nanoparticle tracking analysis revealed a decrease in EV size in both 37°C and 4°C with a slower rate of degradation at 4°C. Notably, multiple freeze thawing of EVs showed no change in EV size indicating that freezing EVs is the best suited method to store EVs [89].

To assess the stability of EVs in plasma, Kalra and colleagues isolated LIM1863 colorectal cell-derived EVs and spiked them in plasma [63]. EV samples were stored with and without protease inhibitors at 37°C, 4°C, −20°C and −80°C and samples were obtained at various time points up to 3 months. Western blot analysis revealed that all recovered EV samples were positive for the EV marker TSG101 indicating EVs are stable at least for 3 months. Consistent with Sokolova and colleagues [89], the results showed that EVs stored at −80°C are highly stable compared to the other storage conditions [63]. These studies confirm that EVs provide a stable environment for luminal proteins and RNA. Even though the stability of luminal protein such as TSG101 is analysed, none of the studies have analysed the stability of EV membrane proteins thus warranting further research.

Isolation of EVs in clinical settings

Although there has been growing interest in the potential use of EVs for disease biomarkers, a general consensus of EV isolation protocol is yet to be achieved [20, 90]. Body fluid-based EV isolation traditionally involves any of the three commonly used methods including differential centrifugation coupled with ultracentrifugation, immunoaffinity pull-down and density gradient separation [19, 20, 63]. In addition to these procedures, several commercial kits have also been developed by vendors and are routinely utilised (Fig. 3). Multiple studies have compared EV isolation methodologies to assess the respective procedures in the context of purity of the isolated population of vesicles and the robustness of the technique. Tauro *et al.* compared commonly used methods to isolate EVs from cell conditioned media and highlighted that immunoaffinity pull-down method isolated EVs containing multiple EV markers [88]. In case of plasma, Kalra *et al.* compared the commonly used methods to isolate EVs and concluded that density gradient separation method yields EVs devoid of protein contaminants [63]. The study employed transmission electron microscopy, Western blotting, atomic force microscopy and MS analysis to confirm that density gradient centrifugation can purify EVs devoid of high abundant proteins thereby reducing the complexity. Similarly, Alvarez and colleagues subjected urine samples to 6 different EV isolation methods and identified a modified ExoQuick-TC protocol as the better method in achieving the highest EV yield and purity compared to standard ExoQuick-TC, ultracentrifugation with or without 30% sucrose cushion or filtration and nanomembrane ultrafiltration methods [91]. Similar to other body fluid analysis, urine EV purity is also affected by high abundant soluble urinary proteins such as THP [91].

While ultracentrifugation is highly utilised for isolation of EVs in research laboratories, it is laborious and time consuming [63, 92]. In addition, patient blood samples are usually limited in volume and may be inadequate to be utilised for ultracentrifugation. This impediment may be overcome by using EV precipitation reagents, such as ExoQuick, which can be easily utilised without ultracentrifugation [91]. Importantly, the EV precipitation methods demand low volume of patient samples. In addition, they are simple, fast and only require a common centrifuge making them ideal methods to be utilised in clinical settings. Even though precipitation methods can isolate EVs that can potentially discriminate between normal and disease samples in the validation phase, it is generally accepted that the purity of isolated vesicles is poor. We emphasise caution in the use of precipitation techniques for discovery phase studies for identification of biomarkers. In such cases, ultracentrifugation or density gradient centrifugation-based methods may be considered as it yields sufficient protein quantities suitable for MS. In spite of possessing several advantages over the conventional biomarker analysis, the field of EVs also face few challenges. A robust method of isolating EVs from complex bodily fluids such as plasma is still lacking. In addition, more analysis is needed to understand the impact of the various methods of sample collection, processing and storage on EV stability.

Conclusions

In the recent past, biomarker analyses have been impeded by the complexity of bodily fluids including plasma, serum, urine and cerebrospinal fluid. Potential disease biomarkers are

often in low abundance and are readily masked by high abundant proteins in the bodily fluids. EVs contain a rich cargo of proteins, RNA, lipids and metabolites which are specifically sorted and often reflect the biological state of the cell type of origin. In addition, EVs contain a lipid bilayer and are highly stable in stored conditions. All these attributes make them ideal choice for biomarker analysis. Isolation of the EV fraction in bodily fluids readily depletes most of the high abundant proteins thereby significantly reducing the complexity of the sample. Hence, EVs-based biomarker analyses have immense potential to be translated into clinical settings. However, as the field is still in infancy, the isolation protocols are still being optimized and more robust methods are needed to harness the true potential in the clinical scenario.

Acknowledgments

Abbreviations

MVB	Multivesicular body
EV	Extracellular vesicle
ILV	Intraluminal vesicle
PSA	Prostate specific antigen
CEA	Carcinoembryonic antigen
CA125	Carbohydrate antigen 125
CSF	Cerebrospinal fluid
SMVs	Shedding microvesicles
NSCLC	Non-small cell lung cancer
SRM	Selected reaction monitoring

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Statement of clinical relevance

Recent extracellular vesicles-based studies have reignited interest in the field of biomarker discovery and validation. Extracellular vesicles, including exosomes, contain a rich cargo of proteins and RNA that are reflective of the host cell type and are selectively packaged. As extracellular vesicles are membranous structures, the luminal contents are protected from degradation by extracellular proteases and are highly stable in storage conditions. Interestingly, an appealing feature of extracellular vesicle-based biomarker analysis is the significant reduction in the sample complexity compared to whole bodily fluids. With these prescribed attributes, which are the rate-limiting factors of traditional biomarker analysis, there is immense potential for the use of extracellular vesicles for biomarker detection in the clinical settings.
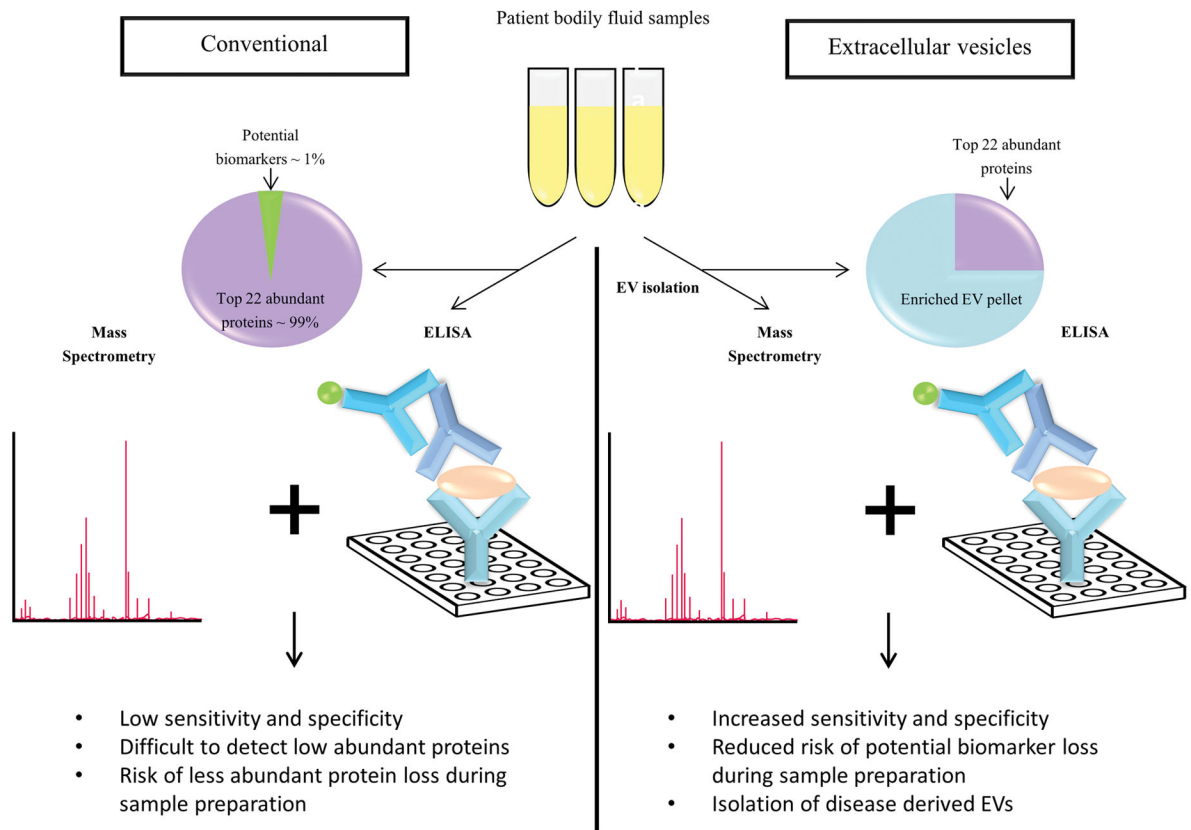
Figure 1. An outline how EVs can be exploited as a potential source of biomarkers in the clinic

EVs can be isolated from cell lines and/or patient samples for identifying potential biomarkers. Following this, shortlisted candidate biomarkers can be validated in large patient cohorts. With the use of EV based biomarkers, there is immense potential for disease diagnosis, prognosis and prediction of response to treatment.
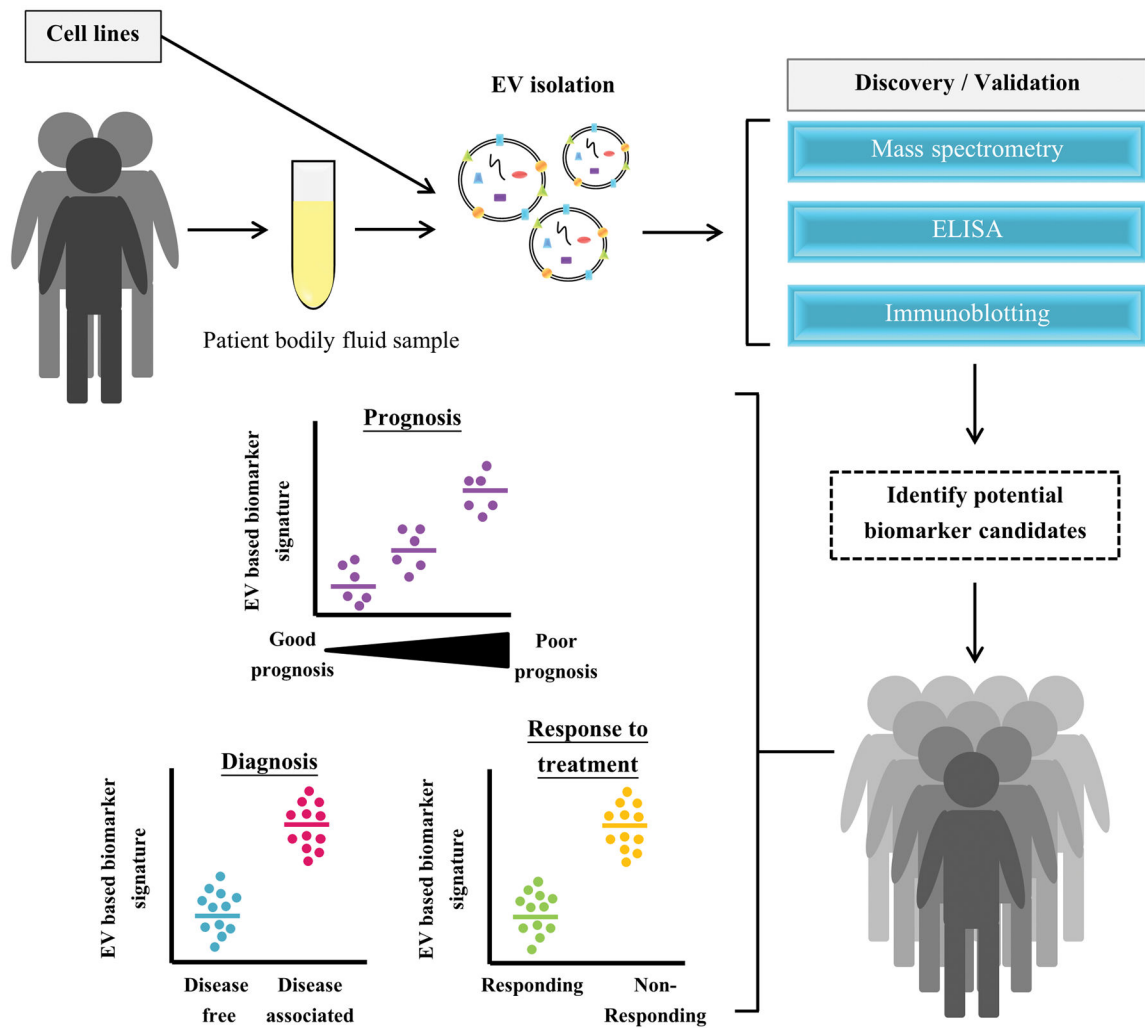
Figure 2. Conventional versus EV-based methods for the discovery and validation of biomarkers
Conventional method of biomarker discovery and validation via mass spectrometry and/or
ELISA are challenged by high abundant proteins in bodily fluid samples thereby hindering
the detection of biomarkers. In contrast, utilizing EVs decreases the complexity of the
sample by depleting the high abundant proteins.
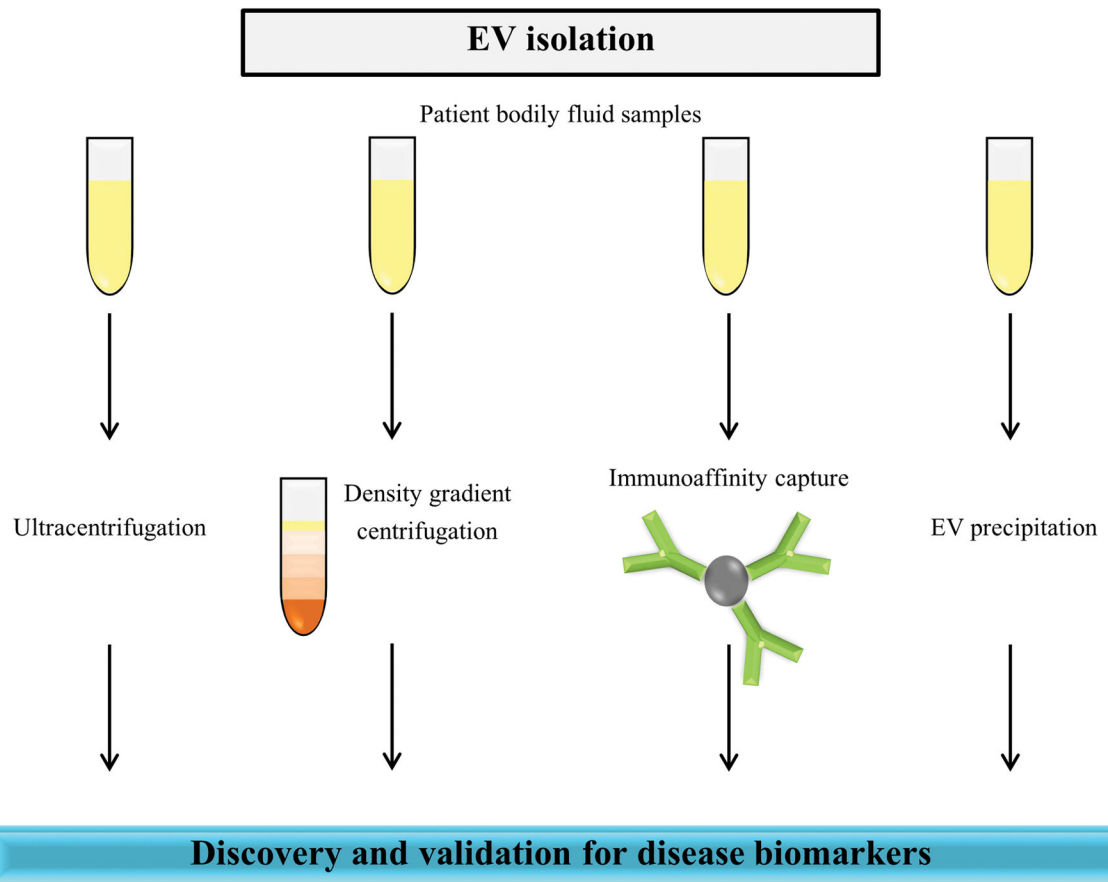
Figure 3. Possible EV isolation techniques for biomarker analysis
Potential EV isolation strategies such as ultracentrifugation alone or coupled with density gradient centrifugation, immunoaffinity capture and EV precipitation can be employed for the detection of EV based biomarkers.

Table 1

Advantages of EVs as potential source of biomarkers

- EVs contain RNA, lipids, proteins and metabolites that are reflective of the cell type of origin. Disease specific proteins or RNA are often detected in EVs.

- The contents inside EVs are protected by the lipid bilayer of EVs.

- They are very stable and can be stored for extended periods of time.

- EV analysis reduces the complexity of bodily fluids thereby aiding in the detection of low abundant proteins.