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From Laboratory to Clinic: Translation of Extracellular Vesicle Based Cancer Biomarkers.

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Abstract

The past decade has witnessed a rapid growth in the field of extracellular vesicle (EV) based biomarkers for the diagnosis and monitoring of cancer. Several studies have reported novel EV based biomarkers, but the technical and clinical validation phase has been hampered by general challenges common to biomedical research field as well as specific challenges inherent to the nanoparticle field. This has led to more common failures than success stories in the biomarker discovery pipeline. As a result, more attention must be focused on the process of biomarker discovery, verification, and validation to allow for translation and application of novel EV based research to patient care. Herein, we briefly discuss the hurdles and potential solutions in EV biomarker discovery and verification and validation, and clinical translation.

Keywords

Biomarkers; Extracellular vesicles; Clinical translation; biomarker discovery; biomarker validation; CLIA

Introduction

Extracellular vesicles (EVs) have garnered much interest in the past decade due to their potential utility as circulating biomarkers for cancer¹. All cells, including cancer cells release small membrane bound nanobodies called EVs. EVs can be classified according to size and biogenesis pathway: exosomes (30-150 nm) are formed by the fusion of intracellular multivesicular bodies with the plasma membrane, microvesicles (100-1000 nm) are formed by budding of the plasma membrane, apoptotic bodies (1000-5000 nm) are produced and released by dying cells, and large oncosomes (>1 μ m) are formed by non-

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apoptotic blebs from plasma membrane²⁻⁴. Tumor derived EVs have been shown to contain tumor specific DNA, RNA and protein cargo which reflect the state of the parent cell of origin^{2,3}. Moreover, EVs have a stable membrane bound structure that protects this cargo from proteases, nucleases and other lytic enzymes. Thus, the ability to detect and stably isolate tumor derived EVs and their cargo in biofluids including urine, plasma and CSF paves the way for their utility as a source of cancer biomarkers and allows for a glimpse into the dynamic molecular composition of cancer cells in time and space⁵. This has prompted a growing body of evidence that suggests EVs may not only play a role as diagnostic markers but also in disease monitoring, early detection of relapse and response to therapy⁶.

Several exploratory studies focusing on small patient cohorts have utilized state-of-the-art proteomic, metabolomic, genomic, epigenomic and transcriptomic approaches to identify novel EV based biomarkers for diagnosing and monitoring various cancers¹. However, very few biomarkers have progressed to clinical utility⁷. As such, there is a large disparity in the number of novel EV biomarkers reported and those currently in clinical use or approaching clinical use. Multiple factors along the line of biomarker development are responsible for the limited clinical application of novel EV based biomarkers⁸.

In this review, we highlight hurdles along the path of EV biomarker discovery to clinical application (Figure 1). We discuss flaws and potential areas of improvement in biomarker discovery, verification, validation, and translation to clinical application. Additionally, we call attention to the role of regulatory bodies in ensuring optimal, sequential, and standardized methods needed to translate EV biomarkers into the clinical realm. We also explore the role of regulatory bodies, including Clinical Laboratory Improvement Amendments (CLIA) and United States Food and Drug Administration (FDA) in commercialization of EV based assays. In light of the challenges of the EV biomarker field, we discuss potential solutions to enhance the translational efforts to bring EV based biomarkers from the laboratory to the clinic.

EV based biomarkers: limitations and pitfalls in translation from laboratory to clinic.

A. EV biomarker discovery

EV Biomarker discovery stems from exploratory studies, which report numerous potential EV based biomarkers that can aid in diagnosis, disease monitoring, and prognostication. Biomarker discovery can occur serendipitously, or as part of a focused study/method or as part of large scale multiparametric screening of several circulating EV cargo. Advances in many areas including genomics, epigenomics, proteomics, lipidomics, metabolomics have made these efforts significantly more plausible. During this phase, limitations in study design, methodologies, sample quality and characteristics, EV isolation platforms, EV cargo analysis, data interpretation and representation can contribute to the failure of biomarkers in reaching clinical utility. Here, we describe the major bottlenecks in different phases of biomarker discovery (Figure 1).

- 1. Study design.** Firstly, the study design should clearly define the clinical need that the particular biomarker would address, i.e. for the purposes of diagnosis,

monitoring of disease progression, or prognostication. It is important that the study has well-defined outcomes and the specific impact the test would have in clinical practice should be clearly stated.

Secondly, studies should be designed with appropriate sample sizes to generate reliable data with significant statistical power. Small sample size can drastically increase biases and have potential for selecting false negatives. Identifying a large number of potential signatures through an underpowered study is not beneficial, as most potential biomarkers are not true and not clinically relevant. For example, sequencing plasma EVs from 5 patient samples and 5 controls can yield to a large number of potential signatures that can distinguish a patient from a control, but the experimental variables are greatly outnumbered by the number of patients included in the cohort. Thus, the chances that a gene signature discovered in this study would be clinically relevant are slim.

Thirdly, selection of appropriate positive and negative controls is the most important, and often the most neglected, aspect of biomarker discovery. Study patients and controls should be from the populations for which the test will be used, and the prevalence of disease in the biomarker study should ideally reflect the prevalence of the disease in the population for which the test will be used. The impact of secondary factors such as smoking, exercise, diets, diet restriction, medications, drugs on EV cargo and number are still unknown and appropriate sample matching of patient and control cohorts can minimize the effects of such confounding factors. Sample blinding is also recommended in study design, as it can remarkably increase the strength of the study by avoiding biases from patient-reported outcomes. Finally, an EV based diagnostic tests should be compared with gold standard diagnostic tests to accurately define the performance characteristics of the test. To this end it is important to note that often times the gold standard test has a lower sensitivity than the newly developed liquid biopsy test, partially due to disease heterogeneity and partially due to lack of technical sensitivity. Thus, statistically powered studies, with appropriate study and control groups, and clearly defined outcomes would enable the discovery of reliable EV based biomarkers that can be translated to the clinic.

2. ***In vitro* and *in vivo* studies.** One of the major challenges of the EV biomarker field is identification and characterization of tumor/disease specific EVs from a vast background of “host EVs” generated from other non-diseased cells in the body. Current EV isolation and analysis technologies have limited sensitivity and specificity in detecting EVs derived from specific cell types in biofluids. Optimizing EV isolation and analysis strategies in *in vitro* studies to determine whether a total pool or a subfraction of EVs are most beneficial to address a specific clinical scenario is ideal. After *in vitro* optimization, EV analysis from *in vivo* studies can further improve separation of target EVs from a heterogeneous background. These vital steps allow for optimization of performance characteristics including sensitivity, specificity, and accuracy before transitioning into verification and validation phases.

3. **Biofluid type.** EVs can be isolated from several biofluids including urine, plasma, serum, saliva and CSF. Careful consideration should be taken when determining the ideal biofluid for sampling in terms of anatomical location, accessibility, and specific biophysical and chemical characteristics all of which can potentially impact the EVs they contain. For example, EV isolation from urine might be best when studying diseases of the genitourinary tract while EV isolation from CSF might be superior when studying neurological diseases⁹. However, the relationship of the biofluid to the disease state is not the only consideration. Although CSF is likely preferable over other biofluids for the study of neurological diseases, the procedure to acquire CSF is invasive, requires highly trained professionals and has potential serious complications. Thus, CSF based EV isolation would not be favorable for neurological disease monitoring, as repeated CSF sampling is not feasible. Hence biofluids such as urine or blood that are far more easily accessible via non-invasive/minimally invasive modalities would be ideal for longitudinal disease monitoring.

- a. **Plasma and serum.** Even samples potentially similar such as serum and plasma yield slightly different readouts in a biomarker assay in patients versus controls, and this variability affects the background and normalizer genes¹⁰. Plasma is usually preferred over serum as platelets release additional EVs during clot formation and can alter the EV composition¹⁰. Serum may be preferential for the study of small RNAs including micro-RNAs^{11,12}. It is recommended to collect blood from subjects after overnight fasting¹³. Physical forces associated with blood draw can cause hemolysis or platelet EV release, hence large diameter, 21-gauge needles are preferred for blood collection for EV analysis^{13,14}. The first 2-3 ml of blood should be discarded¹⁵ and the blood should be collected in plastic collection tubes with the anticoagulant of choice, at room temperature¹⁵. Anticoagulants such as EDTA, sodium fluoride/potassium oxalate (NaF/KOx), or (trisodium) citrate are preferred for preparation of plasma for EV analysis^{13,16}. Heparin based anticoagulants are shown to cause PCR inhibition¹⁷, block EV uptake by cells¹⁸, activate platelets¹⁹ and can drastically influence the biomarker profile and are not ideal for EV based studies. Citrate is the most commonly used anticoagulant and citrate alone or with additives such as dextrose, theophylline, adenosine and dipyridamole prevent platelet activation and release of platelet EVs^{16,20,21}. The choice of anticoagulant is usually dependent on the choice of downstream EV analysis¹³. For example, EDTA is preferred for studying EV RNA²². After collection, the tubes should be positioned vertically during transport and time between sample collection and downstream pre-analytic processing should be minimized to limit the effects on the concentration and functional activity of EVs^{13,23}. Hemolyzed samples should be avoided. When such samples are included, the degree of hemolysis should be measured²⁴ and interpretations should be carefully

drawn²⁵. Pre-analytical processing prior to storage involves centrifugation (1100-2500 x g) of blood at room temperature to remove erythrocytes, leukocytes and platelets²⁶. The samples should be aliquoted and stored at -80°C for future use and thawed at 37°C when required²⁷. Ideally, multiple freeze thaw cycles should be avoided²⁸.

- b. Saliva.** Saliva EVs may be markers for oral cancers²⁹ and diseases such as Sjögrens syndrome³⁰. Most EV studies have utilized whole saliva³¹ and the differences in EV composition based on the gland have not yet been explored. Salivary contents change significantly with eating, drinking, smoking and physical activity^{32,33}. Hence, subjects should remain *nil per os* (with the exception of water) and avoid strenuous physical activity 1 hour prior to sample collection. Subjects should not brush their teeth 1 hour prior to sample collection and should not undergo dental procedures in the previous day. History of any oral diseases should be documented. Diurnal variations in salivary analytes have also been noted, and the time of collection should also be standardized³⁴. For pre-analytical processing, low speed centrifugation or 0.2 µm filters can remove cells, bacteria and food debris. 0.2 µm filters would also eliminate large EVs and can influence the downstream analysis²⁵.
- c. Nasal Secretions.** EVs isolated from nasal secretions can be used to study and monitor respiratory and other airway diseases^{35,36}. Multiple factors, including local tissue disruption (i.e. from nasal swabs, nose blowing, suction, etc.) and seasonal variability (i.e. due to climate differences or levels of inhaled particles like pollen, pollution, or other irritants) may influence nasal secretion EV composition and yield²⁵. Thus, collection of EVs should be as uniform as possible, accounting for each of these factors. Nasal secretion derived EVs can be isolated using gentle nasal saline washes while the patient closes the soft palate to avoid salivary contamination³⁷.
- d. CSF.** The central nervous system (CNS) can be difficult to assess with other peripheral liquid biopsy techniques due to the blood brain barrier, but CSF derived EVs provide a unique window into neurological diseases. Although EVs have been shown to be less abundant in CSF, their detection has been described and used to study CNS cancers other neurologic diseases^{25,38-40}. EV isolation from CSF involves either CSF isolation from the ventricles (i.e during shunt or extraventricular drain placement) or from a lumbar puncture (LP). Specifically for CSF isolated by LP, it is important to avoid contamination with blood, which can affect downstream analysis²⁵. Longitudinal monitoring and assessment of healthy controls can be difficult.
- e. Urine.** EVs isolated from urine can be an excellent window to study diseases of the genitourinary tract. In particular, EVs have increasingly

been used for the study and diagnosis of prostate cancer^{41,42}. Furthermore, collection of urine can be accomplished in large volumes for downstream EV isolation be performed with minimal to no patient discomfort. However, care must be taken to avoid contamination of the sample by bacterial, blood, or epithelial cells²⁵. Of note, Tamm-Horsfall protein (THP), a major component of urine, has been hypothesized to entrap EVs⁴³. Agents like dithiothreitol and other detergents have been used to free EVs from the protein-EV complex, without appearing to alter EV morphology or contents⁴³.

The biofluid type also plays a role in data interpretation. In order to set the appropriate thresholds and properly interpret the results of a test, the biofluid itself along with inter-individual variation, and intraindividual variations (i.e. secondary to collection time differences, medications, etc.) must be considered. Several recommendations have been published regarding the selection of biofluids for EV analysis^{8,44-46}. Thus, biofluid selection and defining specific analytical parameters and thresholds become crucial in biomarker discovery. It is imperative to maintain consistency in sample types during biomarker discovery and validation phase.

4. **Sample quality and characteristics.** The samples from which EVs are isolated are a foundational aspect of EV biomarker discovery. Samples must be high quality and protocols must be consistent, as minimizing upstream variation allows for optimal downstream analysis and data interpretation. As described above, multiple factors influence sample quality, including the method and personnel responsible for sample collection. Technical factors including collection volume, choice of anticoagulant, mixing or agitation, storage and processing temperature, description of transport and pre-processing conditions, whether tube remained upright before processing, exact centrifugation or filtration procedures, degree of hemolysis, possible confirmation of platelet and lipoprotein depletion prior to storage can also influence downstream applications⁴⁷. Furthermore, storage conditions including vials, temperature, freezing and thawing procedures can also affect the integrity of EVs⁴⁸. Parameters such as fresh/frozen sample, number of freeze thaw cycles, storage buffer etc. should also be documented⁴⁷. A recent position statement by International Society of Extracellular Vesicles proposed Minimal Information for Study of Extracellular Vesicles 2018 (MISEV2018) guidelines, which suggested the documentation of pre-analytical patient variables including age, biological sex, current or previous pregnancy, menopause, pre/post prandial status, collection time, exercise level and time of last exercise, diet, body mass index, specific infections and non-infectious diseases, medications, and more. Incomplete disclosure of these sample quality metrics can lead to lack of reproducibility of the biomarkers in different cohorts. Another set of guidelines that can assist in the reporting of these sample quality metrics is the Biospecimen reporting for improved study quality (BRISQ)⁴⁹. These guidelines apply broadly to all biomarker studies and include metrics such as anatomical site, pathology

and clinical diagnosis, type of stabilization, type and constitution of preservative, and details of storage and transport⁴⁹.

Ideally samples as well as controls should come from the same collection protocols to minimize detecting signatures linked to methods and procedures or site of collection. The reverse is also true; a signature should hold true even when the samples are validated in a cohort where the samples are collected under optimal conditions in one single site or the signature would simply suggest that it is a reflection of sample collection rather than of the presence of a disease. Clinical trials provide a great opportunity to generate a cohort of samples balanced and powered enough to generate a robust clinical signature. Our recent study identified protoporphyrin positive EVs in plasma of patients with malignant glioma undergoing fluorescence guided surgery with 5-Amino levulinic acid (5-ALA) as a potential diagnostic strategy to identify and monitor malignant gliomas. Plasma samples used in the study were collected as part of a multi-institutional trial designed to assess the safety and efficacy of 5-ALA as a surgical adjunct to malignant glioma tumor resection⁵⁰. Biofluids collected in a standardized manner can be used for a wide range of biomarker discovery studies. To ensure high quality and to mitigate some of the variation in sample collection, consistent protocols should be in place and personnel should be well trained in sample collection, processing, and storage. However, subtle differences in sample collections will remain between personnel and different institutions. Thus, careful interpretation of results is essential when working with samples from multiple institutes.

Equally important to the handling of the samples is the associated clinical history. Including patient demographics and preanalytical data variables is important as it can minimize biases early in the studies rather than by the time the signature is being validated in larger clinical cohorts. The availability of this information also feeds back into study design- only samples that are linked to data confirming demographics, clinical status, sampling, and handling history can be incorporated into properly designed studies⁸. A clear and specific standardized collection procedure, a pool of well-balanced patient and control cohorts that includes several sites with patient demographics, pre-analytic data variables and a good racial and gender balance provides the study with a higher chance of generating a valuable, clinically significant signature.

5. **EV isolation/separation.** EVs can be isolated/separated from non-EV components of both cell culture supernatant as well as from patient biofluids. Several methodologies for EV separation and concentration have been used across several EV studies. Van Deun et al. showed that approximately 200 distinct isolation methods and 1,000 EV isolation protocols were reported across EV literature⁴⁶. This survey highlights the variability in EV isolation methodologies across various groups during biomarker discovery phase. Methodologies for EV isolation range from differential ultracentrifugation, density gradients, filtration, precipitation, size exclusion chromatography, immunoisolation etc.^{8,51}. Novel methodologies such as tangential flow filtration,

field-flow fractionation (FFF), asymmetric flow field-flow fractionation (AFFF, A4F, or AF4), field-free viscoelastic flow, alternating current electrophoretics, acoustics, ion exchange chromatography, microfiltration, fluorescence-activated sorting, deterministic lateral displacement (DLD) arrays, novel immunoisolation, lipid affinity, precipitation/combination techniques, hydrostatic filtration dialysis, fast protein/high performance liquid chromatography (FPLC/ HPLC), microfluidics have evolved and have variable recovery and specificity of EV population they isolate⁴⁷. Most biomarker studies were performed using ultracentrifugation method, which sediments particles based on speed, time and type of rotor (k-factor)⁵². This technique is limited by high variability in EV outputs, low purity, and the need for technical expertise and expensive instrumentation. Limitations of each EV isolation method have been elaborated on elsewhere and are beyond the scope of this review³. In summary, each isolation platform separates and/or concentrates EVs variably, and mostly non-specifically. This variability feeds into every downstream analysis and can influence the biomarker discovery^{53,54}. Usually the choice of isolation method depends on the required downstream analysis.

In addition to these techniques, there are numerous commercially available platforms/kits for EV isolation. EV isolation kits offer more consistent EV yields compared to traditional methodologies. EV isolation kits manufactured in laboratories that comply with Current Good Manufacturing Practices (cGMP), an FDA regulated organizational system or International Organization for Standardization (ISO), an international regulatory system are listed in Table 1. Biomarkers discovered using these kits offer reproducible EV yields, and studies performed using such kits have a more streamlined translatory capability to the clinic due to the ease of generating reproducible results. Multiple EV isolation techniques, in combination with commercial kits have been used for isolation and/or concentration of EVs from a sample, and some techniques, in combination were shown to further increase yield¹³. This adds another layer of variability to the EV population isolated. Variations in miRNA yields across various isolation platforms provide a window into the heterogeneity of EV populations being isolated through each platform⁵⁵. Researchers should report all the details of the methods to allow reproducibility.

As the EV field continues to grow and evolve, new methodologies/platforms that allow better separation of specific EVs of interest will continue to develop. A gold standard method of EV isolation which allows EV separation with high recovery and high specificity can minimize the biases introduced in EV isolation phase of biomarker discovery. As a result, much attention and effort has been focused in developing improved selective EV isolation techniques to overcome the hurdle of EV heterogeneity.

6. **EV analysis.** Characterization of EVs and their cargo by complementing technologies is important to establish biomarkers. ISEV recommends that each preparation of EVs be defined by 1) quantitative measures of the source of EVs such as volume of biofluid, 2) abundance of EVs, 3) tested for components

associated with EV subtypes and 4) tested for non-vesicular, co-isolated components⁴⁷. Though the biomarker of interest dictates the EV analysis pathway, minimum characterization of EVs can be useful to enhance the reliability of results. Traditional EV analysis platforms include real time PCR, digital droplet PCR, flow cytometry, western blot and ELISA⁵⁶⁻⁵⁸. Novel platforms such as nanoplasmon enhanced scattering (nPES), alternating current (AC) electrokinetic microarray, mass spectrometry, localized surface plasmon resonance (LSPR)-based sensor, imaging flow cytometry etc. have been explored for EV analysis⁵⁹⁻⁶³. The major challenge for any EV analysis platforms is to analyze low input genetic cargo or protein levels for EV biomarker discovery. Optimizing platforms for nanoparticle analysis has inherent limitations in sensitivity of detection of EV biomarker signal⁶⁴. Positive and negative controls are required at every stage of optimization to identify true positive signal. Novel technologies for exclusive nanoparticle analysis are being designed and manufactured to facilitate EV analysis⁶⁵⁻⁶⁷. Studies utilizing such novel platforms should be designed to maximize the sensitivity of biomarker detection and rigorous validation needs to be done to establish the performance characteristics of the platform. Several pharmaceutical and biotechnology companies are invested in generating EV based assays, listed in Table 2, that can aid in detection and/or quantification of EV cargo. To date, there is no FDA approved EV based assay or platform that is approved for clinical use. A few EV based assays, called laboratory developed tests (LDTs), that have been developed in specialized laboratories regulated under Clinical Laboratory Improvement Amendments (CLIA) are available for clinical application (Table 2). Clinical application of EV based biomarking is possible through CLIA validated assays. Further descriptions of CLIA validated assays and LDTs are described below.

Platforms requiring multiple operation steps, prolonged analysis times, low-throughput analysis, expensive equipment and labor intensive protocols will face hurdles during translational phase⁶⁸. Platforms that allow simple, in-expensive, high-throughput, reproducible EV analysis using small volumes of biofluids are required to speeden clinical utilization of EV based assays.

7. **Data analysis and interpretation.** Another pivotal aspect of EV analysis is accurate statistical analysis of the data generated. Data will vary based on biofluid type, sample quality, isolation method employed, and analysis platform. These aspects should be accounted for while interpreting data. Furthermore, datasets will carry an inherent background and identification of true signal depends on appropriate thresholds⁶⁹. Complex EV analysis platforms such as sequencing technologies have complex data analysis and the personnel handling such data should be trained and supervised for reliable and reproducible results⁷⁰. Statistical interpretation must be used to demonstrate a statistically significant difference between the potential biomarker and background noise. Additionally, as most biomarker discovery studies are exploratory and look at large numbers of potential biomarkers in parallel, it is important to account for these multiple comparisons with the appropriate statistical tests. With multiple

comparisons, or multiplicity, higher number of inferences are made that can result in increasing inaccurate conclusions if not statistically accounted for⁷¹.

A well-defined, statistically powered study, with positive and negative controls and, using consistent samples collection, processing, and storage using reproducible EV isolation and analysis protocols and data analysis pipelines will yield clinically useful biomarkers. It is also important to report biomarkers with the same rigorous standards with which they are developed. One set of guidelines that streamlines this process, that includes the bottlenecks described above, is the REporting recommendations for tumor MARKer prognostic studies (REMARK)⁷². These guidelines include metrics such as: markers, study objectives, pre-specified hypotheses, patient characteristics, inclusion/exclusion criteria, assay methods, study design, statistical analysis, and guidelines for interpretation of data and limitations in the context of the pre-specified hypothesis and the field at large⁷².

B. Biomarker verification.

Promising biomarkers should undergo verification to identify the most promising candidates for clinical utility. Verification is identification of true positive biomarkers from a large number of biomarkers identified in the initial biomarker discovery phase. For instance, sequencing studies generate gene signatures with biomarker potential in the context of diagnosis and monitoring. However, not all genes identified are clinically relevant. Therefore, it is imperative to choose relevant EV-biomarker candidates, to verify the genes with clinical implications using established parameters before proceeding to the next phase of biomarker validation. Biomarkers must be verified across several studies in well-defined patient cohorts independent of the original discovery cohorts. Selecting appropriate matched controls based on the target population is critical, as described above. Sample blinding further strengthens the results of the verification phase. Verification not only confirm the potential biomarkers, but also helps in clearly establishing the role of identified biomarkers, i.e. for diagnosis, monitoring and/or prognosis⁷¹. A statistically population balanced study design, that takes into consideration the incidence of the tumor in the general population should be addressed early on to identify the actual relevance of the biomarker. Independent biomarker verification studies, with large well-defined cohorts of samples and matched controls, from multiple institutes can identify robust EV based biomarkers that can be subsequently validated.

C. Biomarker validation

EV assays must also be both analytically and clinically validated. Analytical validation defines performance characteristics of an assay including sensitivity, specificity, accuracy, precision, reportable range, reference interval. Multiple national and international groups including Clinical and Laboratory Standards Institute (CLSI), the International Organization for Standardization (ISO), and the European Committee for Standardization (CEN) have developed consensus standards to address aspects of assay performance characteristics⁸. Clinical validation defines diagnostic performance characteristics including diagnostic sensitivity and specificity which ultimately determines the assay's clinical validity and utility. Biomarker validation should be performed using the same assay, platforms, genes identified in the discovery phase in a large cohort of samples. Validation across several

independent cohorts is more likely to yield robust and reproducible results. Furthermore, sample blinding to the tester allows elimination of bias. Objectively, this rigorous phase of validation helps determine common variables such as age, sex, race and other factors that can potentially impact the biomarker discovery.

1. **Analytical Validation.** An assay is considered analytically validated after several criteria have been tested and defined. These include accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), limit of blank (LOB), specificity, linearity and range. These parameters are pivotal to the assay development and will define the working conditions of this assay in optimal conditions, that is not in a real biological sample but rather in a synthetic setting. Biological and clinical validation will follow.
 - a. **Accuracy.** Accuracy is defined as assay readout of an analyte as compared to the true concentration of that analyte. Often, this is not possible in the liquid biopsy field as gold standard assays do not exist. Furthermore, the EV biology is not clearly defined, and it is largely unknown if cells release a defined number of EVs or EVs with specific cargo characteristics in a predictable way. As such, recovery studies can be performed by spiking in known quantities of EVs/metabolites into controlled buffers/samples.
 - b. **Precision.** Precision determines how closely a minimum of two independent experiments agree. Precision is measured by running multiple replicates of multiple spike-in experiments at multiple timepoints by multiple operators.
 - c. **Analytical sensitivity.** LOB, LOD, LOQ respectively determine how the assay performed in the absence of any biomarker present, the lowest number of a biomarker that is always determined and lastly, the level of biomarker detected that falls within a quantifiable range. These parameters define the sensitivity of the assay.
 - d. **Analytical specificity.** Specificity of the assay will ensure that the assay is measuring the target analyte at the levels truly present in the sample. The specificity of the assay should be assessed by evaluating the performance of the assay in the presence of analytes that closely resemble and likely cross-react with the target analyte. It is important that researchers take precautions and troubleshoot non-specificity and cross reactivity of assay components.
 - e. **Linearity and Range.** Linearity of the assay can be determined using serial dilutions of the target analyte. Such linearity experiments can be used to determine the reportable range of the assay, which represents the span of test results can be accurately measured.
2. **Clinical Validation.** This pivotal phase of biomarker assay development aims at determining the diagnostic accuracy (clinical sensitivity and specificity) and diagnostic predictability (positive and negative predictive values) through testing

thousands of samples in the context of the intended clinical use. Clinical validation differs from analytical validation, as it addresses not only the performance of an assay platform, but also its diagnostic, prognostic, and/or clinical performance. In this phase, a combination of meticulous study design and large sample size can address technical variations, sampling bias, and confounding variables present within patient populations. Study design of a clinical validation study accounts for systematic errors that can lead to false conclusions, such as patient site visit, sample handling or other confounding factors to ensure that they are equally distributed among the different groups. After deciding the internal controls, the criteria for a qualifying sample and the clinical thresholds are established. The thresholds are based on the intended clinical use of the assay, which ultimately determine the diagnostic accuracy and predictability. For example, the ExoDx Prostate IntelliScore (EPI test, Exosomes Diagnostics) is intended as a rule-out test for patients with prostate cancer with PSA levels ranging from 2-10 ng/ml in the initial biopsy setting. A cut off score of 15.6, with a 92-93% sensitivity and 26-34% specificity rules out the possibility of prostate cancer. This is an example where a rule out test should have high sensitivity, meaning that if a patient's score is <15.6, that patient is 92-93% likely not to have prostate cancer and thus should not be tested further. All patients whose score is >15.6 will proceed with the current recommended clinical course (which likely includes a tissue biopsy). In contrast, a rule-in test should have a higher specificity (even with low sensitivity) to definitively identify a true positive, especially when that involves clinical care that deviates with current approved protocols. The EPI test is the first EV-based CLIA validated and clinical available test (Table 3). In 2019, it also received the FDA breakthrough design designation which will lead to a fast-tracked approval process through the FDA.

D. Clinical Translation.

1. **Laboratory Practices and Manufacturing Standards.** Manufacturing laboratories involved in various phases of biomarker development should follow standards that ensures accurate, reliable and reproducible EV assay. Good laboratory practices (GLP) and current good manufacturing practices (cGMP), regulated by FDA and International Organization of Standardization practices are common “established standards” that allow for certification of laboratory standards. Compliance with such practices, though burdensome and expensive, allow for more reliable EV based assays. Researchers interested in biomarker studies ought to consider using kits manufactured using cGMP practices and EV based assays which are CLIA validated, which may optimize clinical application of an assay. However, due to the nascency of the EV field, an appropriate CLIA validated EV assay may not be available. In such circumstances, researchers should consult and collaborate with industry leaders to ensure their work can be translated into a CLIA validated test. These efforts may translate to more economical use of time and resources, including valuable patient samples. Furthermore, this will allow for a more robust arsenal of EV analysis tools to

support this growing field of research and provide a more streamlined transition to clinical validation.

- a. **Good Laboratory Practices (GLP).** Research laboratories involved in EV biomarker discovery and validation should ideally follow GLP, which encompass a set of principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived. Compliance with GLP, ensures uniform, consistent, reproducible, reliable results with quality and integrity. FDA ensures data submitted are true and are reflective of the results obtained during the study. Since GLP practices require strict adherence to the characterization of substances used for experiments, as well as the use of adequate group sizes to allow for meaningful statistical analysis, they provide a high level of scientific rigor that is necessary for the development of a robust, reliable and reproducible EV-based assay in the laboratory setting.
- b. **Current Good Manufacturing Practices (cGMP).** Much like GLP, cGMP is an organizational quality system also regulated by the FDA for both experimental and clinical research that utilizes many of the inherent organizational structures of GLP. Since biomarker discovery is directly implicated in clinical research, following cGMP standards is necessary for reliable and reproducible results. The regulations for cGMP standards ensure the proper monitoring, design and control of facilities that design, develop and manufacture biomarker assays. cGMP standards require a laboratory to have facilities that: 1) are kept in good condition, 2) have equipment that is carefully maintained and calibrated, 3) have qualified and fully trained employees, and most importantly, 4) use operating procedures that are reliable and reproducible. These four requirements of cGMP can be fulfilled by adopting practices that include strong quality management systems, using good quality raw materials, implementing robust standard operating procedures, screening for and meticulously investigating deviations from standard quality, and maintaining reliable testing laboratories. In terms of laboratory practices, standard operating procedures must be developed for each step of test development to ensure proper planning, execution and documentation of experimental procedures. As such, following cGMP standards are considered to be more rigorous, burdensome and expensive to implement and maintain. These cGMP standards are especially important in providing a high level of scientific rigor for clinical studies, which is especially important for the development of a clinically validated EV based biomarker assays.
- c. **International Organization for Standardization (ISO).** cGMP and GLP are standards regulated by the FDA, applying to companies and manufacturing laboratories within the United States. However, most

companies and manufacturing laboratories involved in biomarker discovery are international. These companies and laboratories follow an alternative set of standards known as ISO (e.g. ISO-13485), an international quality management system with cGMP-like regulation. ISO standards are considered to be equally rigorous and just as burdensome to implement, as it follows many of the same regulatory constraints as cGMP standards.

2. **Commercialization.** A verified and validated EV based assay must comply with a set of rules and regulations before commercialization. Commercialization includes marketing an assay for distribution as *in vitro* diagnostics (IVD) which can be purchased and used clinically by laboratories or hospitals. Alternatively, EV biomarker assay can be marketed as a Laboratory developed test (LDT) for clinical use, where the assay is designed, validated, manufactured and used in a single laboratory.

- a. **Development of an EV based *In vitro* diagnostic (IVD).** FDA defines *In vitro* diagnostic (IVD) product as “those reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae”⁷³. IVDs are intended for use in the collection, preparation, and examination of specimens taken from the human body. They undergo rigorous premarket testing for analytical as well as clinical validity. Once FDA approved, IVDs can be sold as a complete kit that laboratories or hospitals can purchase from a manufacturer. They include all the protocols and controls to perform the test independently in a different laboratory. It allows laboratories and hospital who buy the IVD to perform tests with relatively less training and obtain accurate, reliable and reproducible diagnostic results. IVDs can be used very widely and can impact several patients in a variety of settings.

Until recently, there were no EV based IVDs available for clinical use. In 2019, the FDA granted breakthrough designation for ExoDx Prostate IntelliScore (EPI Test, Bio-Techne), a non-invasive EV based urine test measures three mRNAs considered to be important genomic RNA biomarkers that can guide urologists in determining the true need for a prostate biopsy. This is a rule out test is intended for use in men 50 years or older with a prostate-specific antigen (PSA) 2-10ng/mL (“Grey Zone”) presenting for an initial biopsy^{74,75}.

- b. **Development of an EV based Laboratory Developed Test (LDT).** LDTs are specialized subset of IVDs that are designed, manufactured and used within a single, Clinical Laboratory Improvement Amendments (CLIA) approved laboratory. Unlike IVDs which are used by several laboratories, LDTs are performed in a single CLIA validated laboratory by trained personnel in a tightly controlled and regulated

setting for reliable and reproducible results. Hospitals or laboratories can use an LDT only by sending samples to the CLIA certified lab in which the test was developed. Then, this lab performs the test under uniform, controlled, and supervised conditions. As LDTs rely on a single laboratory, ensuring the reproducibility and quality of work flow, equipment and personnel is critical to sustain their reliability of reporting their results.

In the US, LDTs are subject to regulation by the Centers for Medicare & Medicaid Services (CMS) under CLIA, which established quality standards for laboratories conducting testing on human specimens that provide information on diagnosis, prevention, treatment of disease, or the impairment or assessment of health. Such laboratories must be certified by CMS-approved accreditation organizations. Though FDA does not regulate LDTs, CLIA and state specific regulations ensure that clinical laboratories perform rigorous analytical and clinical validation of LDTs. Currently, EPI Test by Bio-Techne is the only CLIA validated EV based assay which is clinically used.

Conclusion

The burgeoning field of EVs has provided a tremendous opportunity for the discovery of disease specific biomarkers that can be utilized for diagnosis, monitoring of progression, and prognostication. However, flaws along various phases of biomarker development have acted as roadblocks along the pipeline from biomarker discovery to clinical application. Accelerating the translation of EV based biomarkers from the bench to the bedside will require researchers to utilize robust methodologies to overcome these hurdles, thus allowing for the establishment of a gold standard of EV isolation and analysis and to identify clinically applicable EV based biomarkers for diagnosis and monitoring of diseases.

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Abbreviations

EVs	Extracellular vesicles
cGMP	Current Good Manufacturing Practices
CLIA	Clinical Laboratory Improvement Amendments
FDA	United States Food and Drug Administration
LDT	Laboratory developed test

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Highlights

- There is a large disparity in the number of novel EV biomarkers reported and those currently in clinical use.
- Multiple factors along the line of biomarker development can be responsible for the failure of EV based biomarkers to reach clinical application.
- We outline the problems and possible solutions along the biomarker development pathway: biomarker discovery, biomarker verification, biomarker validation, laboratory practices and manufacturing standards and commercialization.
- We discuss the role of regulatory bodies including Clinical Laboratory Improvement Amendments (CLIA) and United States Food and Drug Administration (FDA) in commercialization of EV based assays.
- Application of these principles can enhance the translation of EV based biomarkers from the bench to the bedside

Biomarker Discovery	Biomarker Verification	Biomarker Validation		Clinical Translation	
Study Design	Independent well-defined patient cohorts	Analytical Validation	Clinical Validation	Good Laboratory Practices (GLP)	In vitro Diagnostic (IVD)
In vitro & in vitro studies	Matched controls	Accuracy	Clinical Sensitivity	Current Good Manufacturing Practices (cGMP)	Laboratory Developed Test (LDT)
Biofluid type		Precision	Clinical Specificity	International Organization of Standardization(ISO)	
Sample quality & characteristics		Analytical Sensitivity	Positive Predictive Value		
EV isolation/separation		Analytical Specificity	Negative Predictive Value		
EV Analysis		Linearity & Range			
Data Analysis					

Figure 1.
Phases of EV based biomarker development pathway.

Table 1.

Extracellular Vesicle Isolation/Separation Kits

Serial No	Company	Kit Name	Function	GLP	CGMP (ISO-certified)
1	QIAGEN (Exosome Diagnostics)	exoEasy	Purification of EVs from plasma, serum, and cell culture supernatant using column spin-kit	Yes	No (ISO-certified)
2	QIAGEN	exoRNeasy miRCURY Exosome	Purification of RNA from EVs serum or plasma samples using column spin-kit Isolation of EVs from serum/plasma/ cell culture supernatants, urine or CSF using column spin kit	Yes	No (ISO-certified)
3	Exosome Diagnostics	ExoLution Plus	Purification of cfDNA and exRNA	Yes	Yes
4	System Biosciences	ExoQuick	EV isolation <ul style="list-style-type: none"> • High purity • General purpose • Pre-clinical / <i>in vivo</i> studies • Removal of lipoprotein particles from plasma and serum • Defibrinating of plasma step included in kit prior to isolation 	Yes	Yes
5	Norgen Biotek	Exosome Purification Exosomal RNA Isolation Exosome Purification and RNA Isolation Exosome and Free- Circulating RNA Isolation	Purification of intact EVs from serum, plasma, urine, cell culture supernatant, saliva using spin-column proprietary resin Isolation of RNA from EVs from urine and plasma samples using spin-column proprietary resin Combination kit, offers isolation of intact EVs as well as exRNA using spin-column proprietary resin from plasma, serum, urine, cell culture supernatant samples Isolation of pure EV-derived RNA, free-circulating RNA, including microRNA from plasma, serum, urine using spin-column proprietary resin	Yes	No (ISO-certified)

Table 2.

Extracellular Vesicle based Assays : CLIA Validated

Serial No	Company	Kit Name	Function	CGMP	CLIA
1	Exosome Diagnostics	ExoDx Lung(T790M)	A sensitive allele-specific qPCR assay for the T790M mutation on exosomal RNA/DNA and cfDNA with 92% sensitivity and 89% specificity for liquid biopsy in NSCLC and in intrathoracic disease (not yet commercialized)	Yes	Yes
		ExoDx Lung(ALK)	Isolation and analysis of exosomal RNA for five variants of the EML4-ALK mutation from blood plasma		
		ExoDx Lung(EGFR)	Simultaneous isolation and analysis of exosomal RNA and cfDNA to detect activating mutations and T790M mutations from blood plasma		
2	Creative Diagnostics	ExoAssay	Capture and quantifying EVs from plasma, serum, urine, cell culture supernatant against EV-specific markers with ELISA plate	Yes	Yes
		Exosome CLIA	Capture and quantifying tumor derived EVs using specific markers from plasma, serum, urine, and cell culture supernatant with an ELISA plate		
3	Oasis Diagnostics	Pure SAL	Saliva purification for liquid biopsy collects cell free DNA, cell free RNA, exosomes, and proteins	Yes	Yes
		RNA Pro SAL	Collection of miRNA, extracellular RNA, Exosomal RNA, circulating salivary proteins, and Exosomal proteins from saliva		

Table 3.

Extracellular Vesicle based Assays : CLIA Validated and Clinically Used

Serial No	Company	Kit Name	Function	CGMP	CLIA
1	Exosome Diagnostics	EPI test ExoDx <i>Prostate</i> ® (<i>IntelliScore</i>)	Non-invasive urine test that measures three important genomic RNA biomarkers which can guide physicians in determining the need for a prostate biopsy. It is intended for use in men 50 years or older with a prostate-specific antigen (PSA) 2-10ng/mL (“Grey Zone”) presenting for an initial biopsy	Yes	Yes