Review

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Are extracellular vesicles ready for the clinical laboratory?

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Abstract: The diagnostic potential of exosomes and extracellular vesicles (EVs) for liquid biopsies was first demonstrated over a decade ago, but despite a lot of progress in the scientific field there are still very few applications of EVs that are ready for implementation in clinical laboratories for routine diagnostic use. Despite good options for routine isolation of EVs and a wide analyte target space for assay development (incl. RNA, DNA, proteins and intact EVs) assessable by standard detection technologies, the attrition rate in translating biomarker reports in the academic literature to clinical assays is very high. While there are examples of successful development, the largest obstacle to increased clinical utilization is the lack of good biomarkers that can withstand rigid clinical validation, and which make use of the EVs' unique capabilities as a biomarker platform.

Keywords: clinical laboratory; diagnostics; exosomes; extracellular vesicles; liquid biopsy.

Background

Exosomes and extracellular vesicles (EVs) for diagnostic use have come a long way since it was first demonstrated that they can be used to detect clinically relevant molecular markers from biofluids [1]. The most significant advances have been made in basic EV research, especially in the understanding of EV biogenesis, roles of EVs in cell-to-cell communication, and disease mechanisms as well as technical developments of isolation methods and protocols [2–4]. However, despite the progress, there are still very few applications of EVs that are ready for implementation in clinical laboratories.

Blood and tissue cells release a variety of cell-free molecules into biofluids that are suitable as targets for development of clinical assays (Figure 1). Cell-free DNA (cfDNA) is free or nucleosome-bound, fragmented DNA originating through necrotic and apoptotic processes, continuously produced by turnover of otherwise healthy cells [5–7]. In cancer patients, cell-free DNA originating from tumor cells (ctDNA) can constitute a high double-digit fraction (variant allele fraction, VAF) of all cfDNA – especially for necrotic lesions [7, 8] and metastatic late-stage disease [9]. Liquid biopsies using genetic (mutation) cfDNA markers are routinely used to stratify patients to targeted drugs using established biomarkers and good evidence for their clinical utility is accumulating [10]. Further, epigenetic changes (DNA methylation) and measurements of the distribution of cfDNA fragments along the genome (revealing the chromatin structure) are being investigated as the next wave of cfDNA biomarkers. For example, cfDNA fragment analysis is revealing active promoters in the tissue of origin, thereby enabling indirect expression or gene activity analysis [11] and large panels using DNA methylation patterns are at the brink of a breakthrough for early cancer detection with a low level of false positives of around 0.5% [12, 13]. However, for this particularly challenging clinical application, there might be still some development necessary: while a false positive rate of 0.5% is low, the indiscriminate application of a screening test to the general population would lead to staggering absolute number of false positives – about the same number as the currently detected true positive new cancer cases per year [14, 15].

EVs are shed by both healthy and potentially diseased cells, but in contrast to cfDNA, living cells produce EVs through active metabolic processes as means of intercellular communications (e.g., [16, 17]). How exactly this communication occurs during physiological or pathological conditions and if it involves the transfer of molecules between cells is a topic still under intense investigation [18, 19]. For diagnostic purposes, the RNA (mRNA, lncRNA, miRNA, etc.) that is

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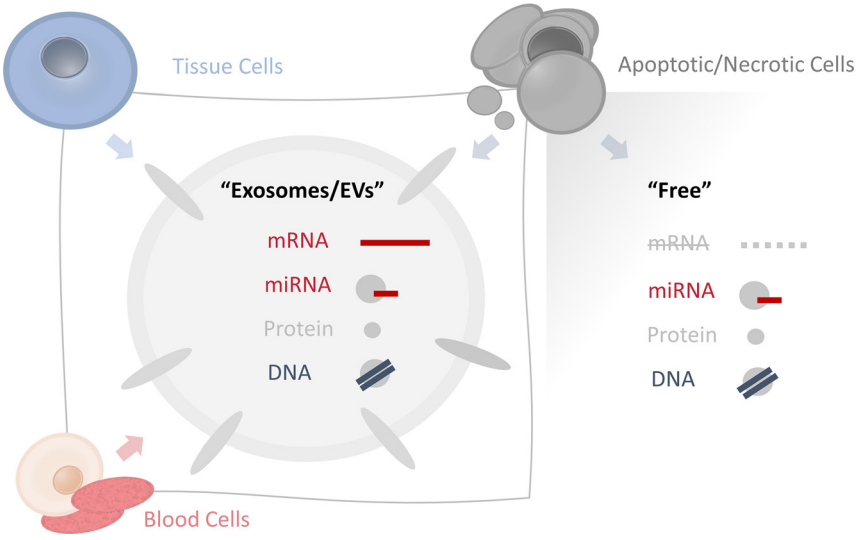

Figure 1: Liquid biomarkers.
A large variety of molecular biomarkers can be found either free-floating and thought to be originating from apoptotic/necrotic processes (right side, "Free") or contained and protected within the double-lipid membrane of EVs that stem from active process of living cells including tissue and blood cells (left side, "EV/Exosomes"). The intracellular/transmembrane proteins and messenger RNAs in EVs are protected from degradation by proteinases and nucleases abundantly present in biofluids.

protected within these vesicles (cfRNA/exoRNA) can be isolated from a biofluid sample [20] and stem from a wide variety of blood and tissue cells [21]. For analytical purposes, EVs can be a source of virtually all molecules present in the originating cells, including protein, lipid, DNA and RNA – but especially long RNAs, protected from the nucleases present in the biofluid, have been utilized for a variety of clinical assays [22–24]. In contrast to the more fragile long RNAs [25], miRNAs exist both inside and outside of vesicles, protected in Ago2 protein complexes from nuclease degradation [26, 27].

EVs also contain DNA, e.g., since they are also shed by disintegrating apoptotic cells [5, 28, 29] and also by processes like NETosis [30], macroautophagy in maturing erythroblasts [31] and egestion of mitochondria [32, 33]. How much of the cfDNA present in a biofluid sample exists as "free" DNA bound to histones and how much is enclosed in EVs is still under active investigation [29, 34, 35].

Since a major challenge of the liquid biopsy field is the general scarcity of molecules available for detection, the material that EVs bring are a welcome addition to any assay that strives for high sensitivity. However, to the clinical scientist, the most interesting aspect of EVs is the extended target space for biomarkers they provide, including intracellular and transmembrane proteins, lipids, metabolites and importantly the means to directly measure expression levels of long non-coding RNA and messenger RNAs.

Pre-analytics and sample preparation for EV collection

When collecting samples for subsequent EV isolation and extraction of analyte (e.g., RNA, DNA, or protein) it is important that pre-analytical treatment of samples is kept consistent (i.e., during sample collection, storage, shipping, and pre-processing).

While the exact type of pre-analytical procedure may be adapted to the downstream assay of choice, there are two parameters commonly important to EV collection: sample collection and pre-processing to exclude larger particles.

For sample collecting, the timing is especially important because EVs are shed by virtually all living cells – and they continue to be so after collection, changing the EV composition while the sample is kept in the test tube. This becomes apparent e.g., in EDTA blood collection tubes, where a variety of white and red blood cells might either burst, releasing cfDNA, or continue to release vesicles that may dilute the vesicles of interest present in the sample. Simply keeping plasma tubes cold, for example, will unfortunately lead to activation of the blood platelets and substantial release of additional vesicles.

In an effort to standardize this step, special blood draw tube types have been developed by some manufacturers, with the promise to halt all biologic processes in the test tube and eliminate the need for immediate processing [36]. However, these tubes still need to be evaluated carefully, for successful stabilization of the target analyte, sensitivity to temperature, degradation of RNA quality, and indiscriminate clumping/aggregation of vesicles by harsh fixatives. Current recommended procedures for blood plasma collection for EV research call for among other things, fasting subjects, special anticoagulants, large bore needles, discarding of the first few mL of blood, keeping the blood tube upright and separating the plasma shortly after collection [37]. Several of these recommendations are not compatible with standard clinical workflow and constitute

a significant barrier to practical clinical adoption. In practice though, choosing and following a defined set of conditions and validating the compatibility with a given clinical assay will suffice. In these authors experience, EDTA plasma, separated within a couple of hours after blood draw will work well for most applications.

Once the plasma sample is separated, the EVs remain stable for days at room temperature or years when frozen and can be stored or shipped without much concern for loss of target molecule integrity. For urine EVs, consensus storage and handling protocols are still being debated [38], but in these authors experience EVs are stable for weeks at 4 °C and days at room temperature, but quickly degrades when subjected to elevated temperatures >30 °C (unpublished data). Urine EVs are stable for years when stored at −80 °C, but some reports suggest significant degradation within a year when stored at −20 °C [39, 40].

Before the samples are ready for processing in the clinical laboratory, they will need to undergo a pre-processing step (pre-filtering or pre-centrifugation) to remove residual cells and debris. EVs are a heterogeneous population of vesicles of difference origins and sizes (Figure 2) and it is important to consider how the sample is pre-processed to remove unwanted particulate matter or cellular debris.

In the reasonable sample pre-filtering range of 0.2–5 µm pore size there is a large size overlap between remaining human blood cells at 7.5–8.7 µm [41], cellular debris e.g., mitochondria at 0.5–3 µm [33, 42] or apoptotic bodies at 0.05–5 µm [43], platelets at 1.5–3 µm [44], and EVs at 0.03–1 µm with exosomes as a smaller subtype generated by the endosomal route around 0.03–0.1 µm [43]. Similarly for centrifugation protocols, which at any given time or g-force will sediment an overlapping fraction of the various biofluid constituents, depending on their size and mass. Most of these components will likely be present in any human sample, and it is important to consider their potential interference with the EVs depending on the choice of isolation and assay. For example, considering that RNA from EVs is by far the minority of the total RNA present in a whole blood tube highlights the strong potential of contaminating the EV fraction with cells or platelets and thereby drastically changing the RNA composition. There is probably no right or wrong filter pore size cut-off, since it depends heavily on the choice of extraction method, detection technology and biomarker type how those parameters may influence the assay outcome but it is important to consistently use the same procedure for any given analysis to avoid diverging results.

Isolation of EVs

From the perspective of the clinical laboratory scientist, it is of lesser importance what the biogenesis or cellular pathway of the EV population is, if it carries the relevant biomarker for a given assay. Similarly, it is important that the extraction and sample preparation can be performed in a robust and consistent manner for EVs to be relevant as a diagnostic substrate.

The traditional method for isolating EVs is by ultracentrifugation. Depending on the specific protocol, this method is cheap and can achieve high yields, but is time consuming and generally low throughput, leading to a push for development of better sample technology in the last 10 years [4, 27, 45].

EV Isolation methods should generally be assessed for their specificity for EVs, repeatability, purity, recovery/yield, turn-around-time, scalability/parallelization, and whether they are good fit to standard lab workflows in equipment, procedure, and price.

Although the choice of isolation method can contribute to the specificity of isolating the EV fraction, the choice of pre-filtration protocol (removal of e.g., cells, cellular fragments, platelets) and downstream assay (e.g., tumor-specific target) is typically far more important for EV-specificity as described above (Figure 2). Consequently, repeatability is probably the most important feature of EV extraction protocols, and any technology that provides this is suitable for laboratory work. This is especially true, for

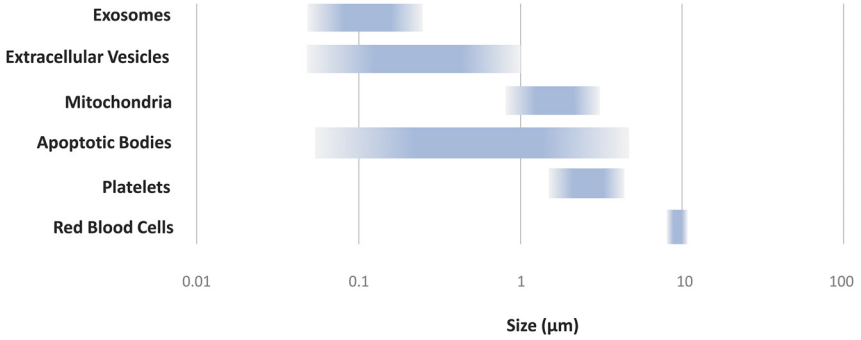

Figure 2: Overlapping size distributions of circulating structures.
There are large overlaps in the size ranges of circulating structures, especially in blood. This spans the range from below 100 nm (0.1 µm) with small extracellular vesicles including exosomes, over cellular debris (1 µm) like mitochondria and apoptotic bodies, up to smaller cellular components like human blood cells just below 10 µm [33, 41–43].

early applications like biomarker discovery since any robust signature should ideally be transferable to another extraction platform when the assay enters clinical laboratory routine. However, the repeatability should be verified in the laboratory, to avoid the generation of misleading data. Highly repeatable exosome extraction is indeed possible, as shown in Figure 3, using two separate extraction methods for two different biofluids.

Still, as for any clinical assay, sample purity must be validated not to contain substances interfering with the downstream assay. Most important is the choice of biomarker, since a marker truly specific for the cells of interest may be less influenced by e.g., co-purified cells or platelets. Equally important is the robustness of the chosen molecular assay, since it may be influenced by e.g., co-purifying RT-PCR inhibitors [46] (e.g., heparin) or excess protein (e.g., blood albumin or IgG), which makes immunodetection of low abundant targets more challenging.

Recovery/yield is also of particular importance in EV based or other liquid biopsy assays since the molecules of interest are usually not very abundant. Apart from the absolute EV capture efficiency, two more aspects are pivotal: maximal sample input and minimal elution volume. The former allows to interrogate a large sample volume and the latter ensures the highest biomarker concentration for the downstream assay.

For the clinical laboratory, processing time, scalability, laboratory fit, and price are of special importance. The most promising technologies that fit this need are polyethylene-glycol (PEG)-precipitation, bind-wash-elute columns, size exclusion columns (SEC), and magnetic beads for immunocapture.

(1) Protocols or kits using PEG precipitation can be used with standard lab equipment, have an easy workflow and will capture EV particles or larger proteins that are present [47]. Co-purification of proteins (incl. Ago2--bound miRNAs) might be a downside, depending on the downstream assay. Also, some procedures require an overnight incubation, which may not be desirable.

(2) Bind-wash-elute columns also provide an easy workflow to capture and concentrate EVs. Simple ultrafiltration columns can isolate EVs from very dilute liquids like urine but will clog easily with denser biofluids like plasma or serum [48]. Commercial columns that utilize

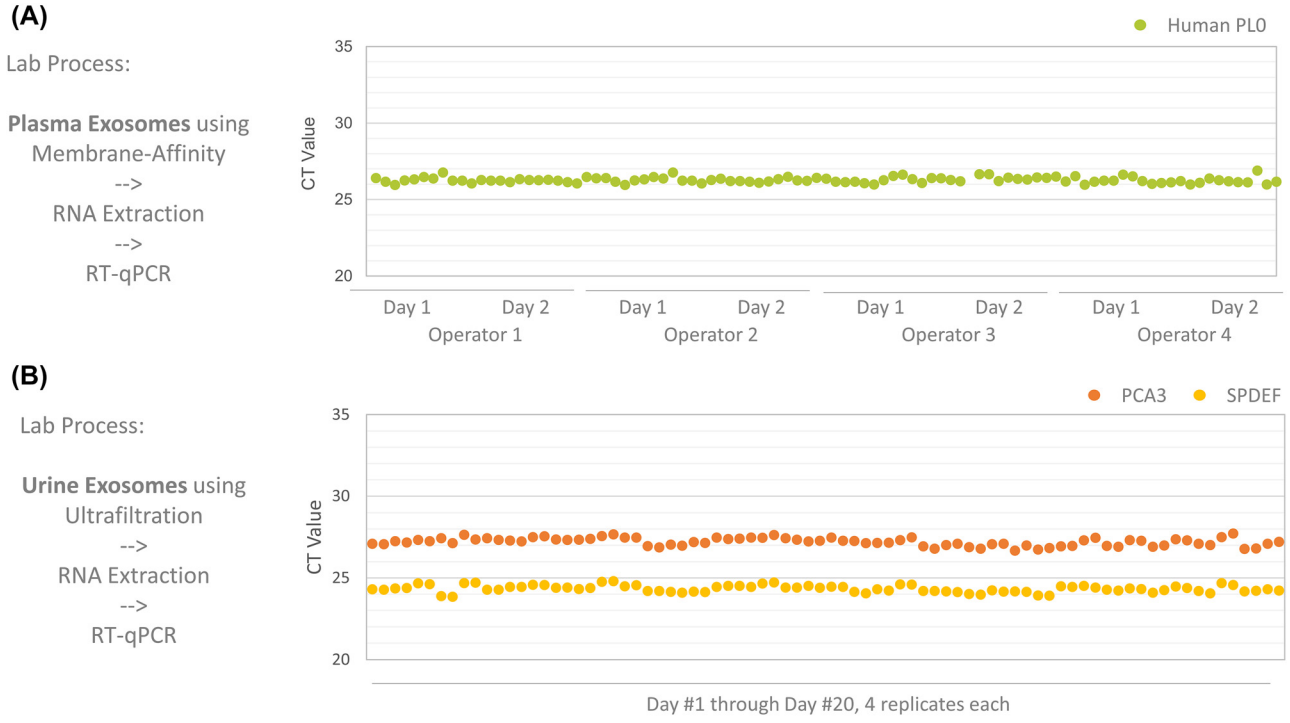

Figure 3: Reproducibility of EV extractions.
Two workflows with independent EV extraction technologies and different biofluids are used in experiments designed to access the repeatability of the procedure. RT-qPCR assays detecting the mRNAs enclosed in the vesicles have a standard deviation of below 0.3 CTs in both experiments, indicating that the extraction variance is low and in the range of assay variance (A) Extraction of plasma exosomes with bind-wash-elute columns using membrane-affinity over 2 days with 4 operators (data courtesy of Exosome Diagnostics, Inc.). (B) Extraction of urinary exosomes with filtration columns over 20 days with 4 replicates each, performed by a single operator [23].

membrane-affinity can be used with a variety of sample types but tend to be relatively costly [27, 45].

(3) SEC columns achieve a high degree of purity and has the additional benefit of creating multiple fractions [49]. This makes them particularly useful for research purposes, but the individual fractions are highly diluted and may be difficult to fit to the assay of choice. Current SEC columns are not easily automated and require operator attention to collect the correct fractions.

(4) Magnetic beads using antibodies against EV epitopes have the potentially most elegant and automatable workflow [50]. Currently, these protocols can be quite expensive due to the antibodies used, but prices should drop significantly with scale. Depending on the specificity of the antibodies, these protocols only capture specific sub-fractions of vesicles which may affect yield and specificity.

In summary, there are several good options to choose from for EV isolation, when carefully considering assay and sample types.

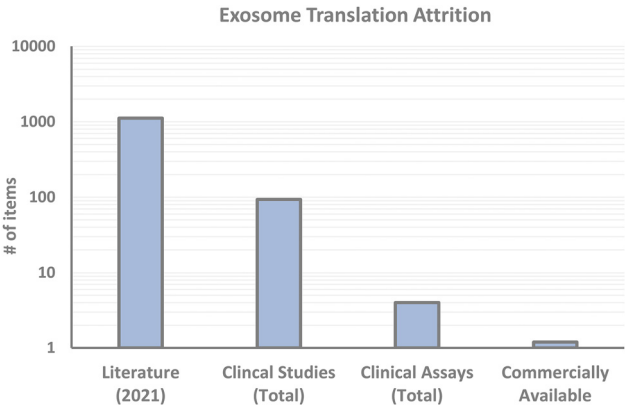

Figure 4: There is a significant attrition on the path from academic research through translation into clinically validated assays. The webpage www.pubmed.ncbi.nlm.nih.gov was searched using the term "(exosomes OR "extracellular vesicles") AND biomarker AND clinical" and restricting to results published in 2021 only (1,120 entries), www.clinicaltrials.org was searched using the term "(exosomes OR "extracellular vesicles") AND (biomarker OR test) AND clinical" and excluding studies labelled as withdrawn, terminated, not yet recruiting, suspended or unknown status (94 entries), and the number of clinical assays (4 tests) and commercially available assays (1 test) was determined through manual review and curation of the results.

Clinical applications of EVs

Despite the intense research activity in exosomes and EVs, the promising developments in sample preparation techniques, and a flurry of articles describing novel EV biomarkers discovered for a wide range of clinical applications, there are still very few clinical diagnostic assays ready for implementation in clinical laboratories and patient sample analysis.

The overall attrition rate when translating exosomal markers to clinical assays become apparent, when comparing the number of published papers on the topic per year (>1,000 in 2021) with the corresponding clinal studies conducted to date (ca. 100 in total) and the number of clinical assays that have reached the stage of clinical validation (4 assays; see Figure 4).

Interestingly, three of the four EV-based assays that have completed or are currently undergoing clinical validation aim to improve clinical care for patients under suspicion of prostate cancer (PCa). PCa is affecting 3.6 million men in the US alone and is the most diagnosed type of cancer with almost 250 thousand new cases in 2021 [15]. Since PCa is a "cold" tumor without many somatic mutations [51] and the most frequent mutation is a translocation giving rise to the TMPRSS2:ERG RNA fusion transcript [52], PCa is difficult to detect in a liquid biopsy using cfDNA. Exosomes and EVs provide the opportunity to apply innovative RNA or protein expression biomarkers. The ClarityDx™ System uses micro flow cytometry to count exosomes in a blood draw that are positive for three biomarkers: GHSR (ghrelin receptor), PSMA (prostate-specific membrane antigen) and polysialic acid ([53–55], NCT03957252). The Sentinel™ PCC4 test relies on an expression signature of 442 small non-coding RNAs (sncRNA) in urinary exosomes ([56], NCT04100811, NCT04661176) and the ExoDx *Prostate(IntelliScore)* test uses a 3-gene urine exosomal long RNA signature of ERG (V-ets erythroblastosis virus E26 oncogene homologs), PCA3 (prostate cancer antigen 3), and SPDEF (SAM pointed domain containing ETS transcription factor) [57]. The fourth EV test currently undergoing clinical validation is a variation of the Sentinel™ test, using a 280 sncRNAs signature, for detection of bladder cancer (NCT04155359).

The ExoDx *Prostate(IntelliScore)* has been commercial availability in the US since 2016 and is being reimbursed by Medicare based on the extensive clinical evidence in clinical validation and -utility studies of over 2000 patients ([58–60], NCT03235687, NCT03031418, NCT04720599, NCT02702856). The test utilizes bulk isolation of urinary exosomes by ultrafiltration, followed by RT-qPCR of the three RNAs that combine in the "EPI score", which is correlated with the risk of significant prostate cancer. The test was recently certified

as a CE-marked *in-vitro* diagnostic (IVD) test and revalidated in a European population [23], making it the first exosome-based test to reach this milestone and demonstrating that EV isolations can successfully be used in routine clinical laboratory diagnostics.

The challenges ahead for EVs in the clinical laboratory

The main obstacle for EVs in the clinical laboratories is that other than the EPI test, there are no IVD products available and very few known biomarkers that can serve as the basis for successful laboratory developed tests (LDT).

EVs represent a new sample fraction for liquid biopsy analysis, alongside whole blood, plasma, serum, cfDNA, peripheral blood mononuclear cells (PBMCs), circulating tumor cells (CTCs), platelets, urine, urine cell pellets, cerebrospinal fluid and probably a few others.

The absolute amount of EVs themselves in a biofluid sample has not (yet?) been shown to be a biomarker for anything. Rather, similar to cfDNA, EVs provide a new target space for biomarkers and the task of discovering, developing and validating a bona fide clinically relevant biomarker ready for clinical use is long and perilous as evidenced by the journey of the ExoDx *Prostate (IntelliScore)* test. The assay was first described in 2015 [57], the first clinical validation study published in 2016 [58], a second validation study in 2018 [58], a clinical utility study in 2020 [59] and finally launched as a CE-marked IVD in 2021 [23].

The use of cfDNA in clinical diagnostics has been well established through years of research and development and is currently being integrated into the standard of care [10, 60]. Still, cfDNA-based assays to date usually have the relatively easy task of determining the presence or absence of known somatic mutations whose clinical relevance has already been established in tissue samples. If the mutation signal is above a pre-defined threshold, there is little ambiguity about the laboratory result and the usefulness to the clinician is given by the corresponding targeted drug [61].

Most EV biomarker candidates currently under development are not established markers, but rather focus on the EVs unique advantage to provide proteins and RNA. These biomarkers usually cannot be detected as easily as a somatic mutation but rely on the exact quantitative determination of the abundance of the markers. Moreover, many assays are based on analytes that are not unique to the EV fraction and/or tumor (e.g., a wildtype mRNA or protein), subjecting them to potentially significant sources of error during sample collection, pre-analytics and sample processing. Even the smallest amount of cross contamination with cells or platelets will make the abundance of many RNAs and proteins increase dramatically. We recently found the platelet fraction from whole blood to contain 250 times more wildtype BRAF mRNA (340,000 copies/mL) than the EV fraction (1,300 copies/mL), impeding detection of any mutated BRAF transcripts of tumor origin potentially present in the platelet fraction, whereas the BRAF V600E mutation was easily detectable in the corresponding EV fractions [62]. Any quantitative EV biomarkers based on analytes that are not clearly of tumor origin (e.g., RNA expression like in the EPI test) will have to be very robust to such contaminations from cells or platelets, posing a significant hurdle to the development of clinical assays.

RNA fusion transcripts present an example of a unique use of RNA from EVs, since biomarkers with a high specificity to tumors, like somatic mutations and rearrangements, are easily detected and relatively resistant to contamination from other biofluid fractions. Large next generation sequencing panels, used in the clinics to detect the pathological fusion event on the RNA level, eliminate the need to wastefully cover the complete intronic and often repetitive sequence space in the DNA where a breakpoint might have occurred [63]. Just as detection of point mutations has successfully been translated to liquid biopsies using cfDNA, it seems straightforward to translate detection of RNA fusions (e.g., NTRK, EML4-ALK) by using the cfRNA in the EV fraction. Several examples utilizing this approach have been published [1, 22, 64] or are under clinical investigation (NCT04499794).

Another promising biomarker approach is the use of EV surface proteins. Similar to CTCs, EVs can be captured and detected using their membrane surface markers. Technical solutions for detecting surface proteins on intact EVs are especially interesting, since they have the potential to interrogate multiple markers on the same vesicle and perhaps even circumvent the need for prior purification. Direct detection of EVs by flow cytometry is still not routinely possible because the size of most EVs is below the detection limit of forward scattering detectors in standard flow cytometers. However, capture of EVs on beads, followed by detection of unique surface markers in a “Sandwich” EV flow cytometry assay seems like a promising approach, which is readily available for research use [65]. More specialized flow cytometry approaches are already evaluated in clinical studies, including the micro flow cytometry (μFCM) used by the ClarityDx test [53, 54] and

antibody-based proximity ligation (exoPLA) with rolling circle amplification to create a detectable signal [66] (NCT03694483). As a well-established technology, ELISA is currently being investigated in a field, where EVs themselves are part of the disease biology: some tumors have been reported to specifically secret EVs with PD-L1 receptors on their surface to blunt the hosts immune response [67, 68]. Detection of exosomal PD-L1 is therefore under investigation as a predictive marker for response to checkpoint inhibitors that target the PD-L1 receptor [68, 69].

It may be interesting to note, that EVs can also provide access to intracellular proteins that are otherwise inaccessible to analysis. Specifically, the phosphorylation status of signaling proteins, frequently involved in disease biology, may develop into a new class of liquid biopsy biomarkers only accessible through the EV fraction [70, 71].

Overall and especially for biomarker discovery, it is important to keep in mind that EVs provide a completely new target space with markers that exceed somatic mutations and while current applications and studies are heavily focused on oncology, EVs provide an opportunity to translate any RNA/protein expression biomarker that has been identified in any medical indication (Alzheimer's disease, diabetes, cardiovascular disease, etc.) into a liquid biopsy test.

In summary, clinical assays based on EVs need to overcome a few points before becoming a mainstay of the clinical lab:

(1) Sample collection: easy. The main benefit of any liquid biopsy assay is the ease of sample collection. Compared to tissue, the sample collection is generally easy and straightforward. The collection needs to fit with the clinical routine, but the task seems reasonably straight forward.
(2) Pre-analytics: difficult. Many unanswered questions and a field of active development. Although especially the smaller EVs are relatively stable, this is not necessarily true for larger vesicles. Depending on the individual biomarker, this will need to be validated on a case-by-case basis. For blood samples, a more important confounding factor than stability is the potential for creation of additional EVs by activation of platelets in the pre-analytical sample. Blood tubes for sample preservation still need careful investigation, but tightly controlled standard operation procedures may suffice for some applications.
(3) Sample extraction: easy. Many methods for EV isolation and analyte extraction have been developed, including some that are suitable for routine clinical laboratory use.
(4) Analyte detection: easy. Although present in low amounts, EVs contain standard analytes that can be detected by standard clinical laboratory techniques such as (RT)-qPCR, ELISA, FACS, microarrays, Next Generation Sequencing or Western Blotting. Protocols may need optimization to robustly detect the limited number of molecules generally available in liquid biopsies.
(5) Biomarkers: Missing. The largest hurdle preventing EVs from translating into routine clinical laboratory assays is the lack of robust biomarkers that answer a defined medical question. This is partially due to the challenging, quantitative nature of biomarkers that are pursued in EVs and will need significant development effort in the years to come.

So, are extracellular vesicles ready for the clinical laboratory? We are inclined to answer "yes", with due consideration of the above-mentioned caveats. EVs are ready to be used as a biomarker platform and can fulfil the stringent criteria that are expected from clinical assays [72] – but what is largely missing are the biomarkers and assays that successfully make use of this new target space. There is little evidence of strong efforts in any area beyond prostate cancer for now, but this is likely to increase in the future, given the recent proof of principle in this particularly challenging indication.

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Competing interests: Daniel Enderle and Mikkel Noerholm are former employees of Exosome Diagnostics and developers of technology mentioned in this manuscript, now commercialized by Exosome Diagnostics and Qiagen. Authors state no other conflicts of interest.
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