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Title: Utilising extracellular vesicles for early cancer diagnostics: benefits, challenges and recommendations for the future.

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Abstract:

To increase cancer patient survival and wellbeing, diagnostic assays need to be able to detect cases earlier, be applied more frequently, and preferably before symptoms develop. The expansion of blood biopsy technologies such as detection of circulating tumour cells and cell free DNA has shown clinical promise for this. Extracellular vesicles released into the blood from tumour cells may offer a snapshot of the whole of the tumour. They represent a stable and multifaceted complex of a number of different types of molecules including DNA, RNA and protein. These represent biomarker targets that can be collected and analysed from blood samples, offering great potential for early diagnosis. In this review we discuss the benefits and challenges of the use of extracellular vesicles in this context and provide recommendations on where this developing field should focus their efforts to bring future success.

Utilising Extracellular Vesicles for early cancer diagnostics: benefits, challenges and recommendations for the future.

Early diagnosis is accepted to be one of the key strategies for improving patient outcomes and is shown to be associated with longer survival in many types of cancer. For example, in ovarian cancer, if disease is detected when it is confined locally (stage 1), five-year survival is as high as 93% [1], but this falls to 26.9% and 13.4% for stages 3 and 4, respectively [2]. Similarly for lung cancer, five-year survival rates fall from 56.6% for patients diagnosed in stage 1, to 12.6% and 2.9% for those diagnosed in stage 3 and 4 respectively [3]. The application of new and more sensitive approaches to early detection could be more important for patient survival in some specific cancer types. In breast cancer, for example, there is growing evidence that metastasis, generally considered a characteristic of late stage disease, can actually occur at a very early stage in disease development, and micro-metastases may be present in patients at the time of diagnosis which do not become clinically apparent until much later [4,5].

The World Health Organisation emphasises the importance of promoting early diagnosis in reducing the need for invasive and expensive treatments, and lowering mortality and morbidity associated with later-stage cancer [6]. Moreover, the United Kingdom's (UK) National Health Service announced in their January 2019 'Long Term Plan' their ambition to increase the proportion of cancers diagnosed early - that is, at stages 1 or 2 - from around 50% to 75%, leading to an estimated 55,000 additional UK cancer patients surviving for five years following their diagnosis [7]. Part of this drive is built on earlier detection through greater public education campaigns such as 'Be Clear on Cancer', but much is also focused on faster, earlier, more accessible and personalised diagnostics, captured in the 2019/20 Rapid Diagnostic Centres Vision, a programme for transformation in cancer diagnostic in UK healthcare [8].

In order to achieve the ambition of a more rapid, sensitive and personalised approach to diagnostics, there is a need for a significant step change in scientific technology. The recent explosion in the understanding of the roles of extracellular vesicles (EVs) in normal physiology and in disease processes, including cancer, in tandem with the genomic technology revolution, provide great promise to fill this gap. This review provides a brief introduction to the potential of EVs as early diagnostics in cancer, with a focus on the sensitivity of cancer-specific EV detection in blood, and the technical challenges that need to be overcome. We then propose recommendations for the implementation of EV detection in blood-based assays for early detection of cancer.

An introduction to extracellular vesicles

Extracellular vesicles is a collective term for membrane-bound vesicles that are synthesised and released from cells in a range of sizes. The most widely discussed EVs are commonly known as exosomes (30-150nm). They are assembled and released from the multi-vesicular endosomal system. Microvesicles (MVs) are slightly larger (100-1000nm) and arise as they are pinched off from the plasma membrane. Apoptotic bodies are much larger in size (50-5000nm) and are released by dying cells. A range of cancer associated EV types have been described, including large oncosomes [9,10] estimated between >1000 nm to >10,000 nm and

exomeres, a much smaller non-membranous nanoparticle at an estimated 35nm [11]. This is important in diagnostics due to differences in scientific approaches required to extract and analyse various vesicles [12]. An updated position statement by the International Society of Extracellular Vesicles (ISEV) was published in 2018 [13] and discusses some of these challenges.

EVs carry cargo of DNA, RNA, proteins, lipids metabolites, and even fragments of organelles, and represent a cellular communication system that is highly conserved through evolution, from prokaryotes to humans. They play a myriad of functional roles in normal development and physiological processes through cell-cell communication via delivery of their cargo to recipient cells, reviewed by Yáñez-Mó *et al* [14], including increasing evidence of their functioning in disease processes, reviewed by Becker *et al* [15]. There is currently much interest in the detection of EVs and their cargo as biomarkers of cancer, including their potential for developing new approaches to early detection. Great advances in molecular imaging and 'omics' technologies over the past decade has allowed the detailed profiling of EVs. Their presence has been reported in all body fluids tested, including in blood [16]. EVs in the blood have been shown to represent a proxy for the tissues that they are released from, which is reflected in their cargo, giving insight into the donor tissues characteristics, including progression to malignancy [17,18], which are paralleled by changes in EV quantity or contents [19]. Hurwitz *et al* have shown that the proteomics of a common set of cancer cell lines covering 60 of the many cancer types from the United States National Cancer Institute shows that EVs have similar content to their donor cells, supporting the case for their use in blood diagnostics [20].

The benefits of extracellular vesicles over current blood biopsy diagnostic methods.

The potential to detect disease biomarkers in blood has the appeal that the sample is truly systemic, containing elements derived from, and pathologically reflective of, all bodily tissues. Moreover, blood sampling is cost effective, minimally invasive, technically simple, and can be performed repeatedly across the patients care pathway, allowing real time tracking of the changes in the tumour and supporting clinical decisions on the most advantageous therapy plans. The blood EVs are reasonably well preserved frozen before analysis making clinical management, sample storage and biobanking easier for diagnosis and long-term research projects.

In recent years, much emphasis has been placed on detection of circulating tumour cells (CTCs) and cell free DNA (cfDNA) derived from cancer cells, but their detection and analysis from blood can be problematic, as recently reviewed by Salvianti *et al* [21]. For example, CTCs occur at very low frequency in blood and degrade within a few hours making their detection problematic in clinical practice, and raising technical challenges around transport and storage for later analytics [22.] In comparison, EVs offer the advantages of longer-term stability [23,24] and storage. Although the biomarker concentration per EV is very low compared to per CTC, EVs are present in very high numbers in the blood, reported to be from around 10^9, to 10^{12} particles per milliliter [25,26]. This count variation between individuals is dependent on a number of pre-analytical variables and technologies used for counting[12]. It is still suggested that the number of EVs in the blood greatly exceed that of CTCs, raising the possibility that EVs could provide a more sensitive assay system, for early diagnosis such as the single EV level technologies mentioned later. For example, Nanou *et al* report that EVs derived from epithelial cells specifically (EpCAM and CK positive) were present in numbers of at least an order of magnitude greater than CTCs in the blood of patients with breast, lung

and colorectal cancer[27]. There are concerns with the low numbers of CTCs for detecting relapse. In a two year follow up of 1087 breast cancer patients using a common CTC clinical assay testing the 101 patients with relapse, only 36 were positive CTC status [28]. Though, the benefit is not exclusive to the analysis of EVs on their own, a study on the diagnosis of pancreatic ductal adenocarcinoma showed the addition of glypican-1 positive blood EVs to CTC detection in patients increased positive diagnosis from 32% to 64% [29].

Similar limitations apply to the detection of cfDNA which occurs at very low abundance and is subject to fragmentation, making detection and analysis difficult [30]. EVs are known to be abundant in RNA, thought to be protected from the harsh environment of the blood by the EV membrane. Although very different analytes, the RNA contents of EVs alongside cfDNA diagnostics in lung cancer. One research group shows that the addition of EV RNA to cfDNA increased detection of copies of the *EGFR* activating mutations by as much as tenfold [31]. An advantage of most EVs is that they are released from living cells, therefore reflecting the live dynamics of the developing, including early stage, where cfDNA tends to be derived from dead cells. Research has shown that cfDNA used in tandem with small EVs could increase the sensitivity of cancer liquid biopsies in advanced cancers and over time correlate better with treatment outcome than cfDNA biomarkers alone [32]. In a study of serial blood samples taken from 34 pancreatic cancer patients it was shown that *KRAS* mutations in the EV DNA increased with disease progression; this was not seen in cfDNA measurements [33]. There may be specific cases where EVs have a benefit over cfDNA. In a study assessing relapse detection for early stage breast cancer, they showed that metastatic relapse was detected in 96% of the patents when the relapse was outside the brain, but for only 17% of those with brain-only metastasis [34], exosomes have been shown to traverse the blood brain barrier in brain metastasis from breast cancer *in-vivo* [35] offering the potential for the systemic testing of brain tumours out of reach for cfDNA and CTCs. That said there is advantage in adding EV data to the CTC and cfDNA data, that can be collected from the same blood sample.

The potential of EVs as early detection biomarkers

Much of the current research focus in EVs as early cancer diagnostics has been on increasing the sensitivity of the analysis - both in terms of the ability to identify a greater proportion of true positive samples, and also of being able to analytically detect the EV biomarker at very low concentrations in the sample. The unique structure of EVs and the diversity of cargo that they carry provide features that technically facilitate both. Below are some examples of common biomarker analyte types that have shown potential for early diagnosis using EVs.

The profiling of RNA in EVs was a key turning point in revealing not just the mechanisms by which EVs mediate cellular communication, but also in determining that EVs could provide a diagnostic tool for cancer [19,36,37]. An early paper on the identification of an EV EGFR mRNA mutation as a diagnostic in the serum of glioblastoma patients, this team later led to the first commercial EV diagnostic to reach the healthcare market with a focus on prostate cancer[38]. This is not just for coding genes, as one group find a collection of 6 long-non-coding RNAs can differentiate between healthy and 15 stage I/II colorectal patient plasma EV samples [39]. More recently this has also been seen in other types of RNA like circular RNA [40]. EVs containing circRNA-SORE has been implicated with drug resistance in hepatocellular carcinoma, and at high levels correlate with poor patient survival. This is being investigated as its directly linked *in-vitro* with YBX1, a protein seen at significantly lower levels in early stage cancer [41].

MicroRNAs are a key focus in EV diagnostics. Jin *et al* demonstrated how a four-microRNA signature of let-7b-5p, let-7e-5p, miR-23a3p, and miR-486-5p in plasma EVs showed 80.25% sensitivity and 92.31% specificity in distinguishing early-stage non-small cell lung cancer (NSCLC) patients from healthy individuals. Moreover, other microRNA signatures could distinguish adenocarcinoma and squamous cell carcinoma among the identified NSCLC patients [42]. EV cargo has also been suggested to predict treatment response early. For example, in addition to being able to distinguish patients from controls, upregulation of the microRNA 17/92 cluster in plasma EVs has been associated with patient response to adjuvant chemotherapy in rectal cancer and its post treatment prognosis [43]. EV RNA cargo molecules make ideal biomarkers for early cancer detection, both because they reflect live dynamic changes in the cells that release them due to the relatively short half-life of RNA, but also because they can be detected using inexpensive, reliable and widely available technologies such as qPCR and digital PCR. This also means that the faint signal can be amplified millions of times that found in the original sample, realising very high sensitivity [44]. In addition to the ability to greatly amplify signal using standardised biotechnology tools, there is evidence that the enclosing EVs membranes offer signal stability for RNA. Cheng *et al* show that EVs provide a protective barrier for microRNAs, preserving the signal against systemic RNAse [23]. There is also a growing interest in the diagnostic promise of extracellular RNA naturally protected by protein complexes like Argonaute-2, independent from EVs [45], as reviewed by Li *et al* [46].

DNA is also an important nucleic biomarker in this field. Balaj *et al* were the first to show that tumour cells release micro vesicles containing DNA. They found DNA encoding the *c-Myc* oncogene and retro retrotransposons in EVs released from both cultured medulloblastoma cells and in the serum of tumour-bearing mice [47]. In 2014, the use of EV-derived DNA in cancer diagnosis was expanded by a number of other groups. Lázaro-Ibáñez *et al* confirmed the presence of genomic DNA fragments in the EVs in the circulation of prostate cancer patients plasma and those EVs released by cell lines, and that DNA mutations could be seen of common cancer genes like *MLH1*, *PTEN* and *TP53* in the EVs of the cell lines [48]. Kahlert *et al* demonstrated that EVs derived from pancreatic cancer cell lines and the serum of patients with ductal pancreatic adenocarcinoma both contained double-stranded DNA carrying *KRAS* and *p53* mutations and importantly that DNA derived from all chromosomes was detectable [49]. Lee *et al* also show EVs containing double stranded DNA across the genome from brain tumour cells, that are taken up by recipient cells [50]. Thakur *et al* also reported EV-derived double stranded DNA representative of all chromosomes, while demonstrating *BRAF* mutations in EV-derived DNA from several melanoma cell lines and from serum of mice implanted with melanoma cells, as well as epidermal growth factor receptor (*EGFR*) mutations in EV-derived DNA from NSCLC cells. Interestingly, they show the methylation level of the EV-derived DNA was shown to be similar to that of the original genomic DNA [51]. These studies suggest that EV-derived DNA could potentially reflect the status of cells across the entire tumour, across the genome, allows analysis of specific cancer-associated mutations. Moreover, importantly, they demonstrate the potential for combined analysis of methylation, and this was also reported in a recent study using gastric fluid EVs for early detection of gastric cancer [52]. They raise the possibility that not only can commonly employed, inexpensive and robust nucleic acid technologies be used to massively amplify signal in blood/body fluid biopsies for more sensitive sampling, but that such samples can be multiplexed to offer a much more complex analysis than was previously appreciated.

Although it does not have the same simple amplification benefits of nucleotide technologies, EV protein-based biomarkers can be associated with stage, treatment response and prognosis of cancers. One example is Fibronectin on the surface of EVs from breast cancer patient

plasma, has been shown to be elevated compared to healthy individuals at all stages, including the early stages [53]. Modified proteins have also shown promise. For example, the presence of the proteoglycan glypican-1 in EVs derived from serum has been shown to distinguish early from late-stage pancreatic cancer patients [54]. Niu *et al* show that serum EV levels of alpha-2-HS-glycoprotein, extracellular matrix protein 1 and carcinoembryonic antigen showed an AUC of 0.911 for 35 early non-small cell lung cancer patients versus 46 healthy individuals [55].

Some of the main challenges for early detection are to identify patients at a premalignant or latent stage in disease development, and to identify patients with apparently localised early disease. In the case of potentially screening blood and serum for a biomarker associated with early cancer, identification of the tissue origin of the disease signal would be hugely beneficial. There are indications that EVs may have the potential to address all of these issues. As an example, Mathivanan *et al* [56] built on early work by Mallegol *et al* [57] and showed that EVs released from intestinal epithelial cells exhibit markers that identify their cell of origin, including A33, a molecule that is restricted to intestinal epithelium. They demonstrated that it was possible to employ immunoaffinity capture to isolate and enrich EVs expressing A33. Then, in a comparative analysis, in addition to identifying a range of markers that distinguished EVs released by the colon cancer cells by carcinoembryonic antigen (CEA), they were also able to establish a subset of markers common to all EVs derived from epithelial origin. These include epithelial cell surface antigen (EpCAM) and keratin18, which are now commonly used to enrich EVs of epithelial origin from blood samples where they are present in only small quantities, thus providing powerful signal amplification by EV enrichment. It has been reported that a general increase in the number of epithelial-derived EVs in the blood is indicative of the presence of tumour [58]. A novel example of this approach is EV enrichment based on binding and extracting the EVs using the protein LIM1215, suggested to enrich for colon cancer-derived EVs [56]. This approach of enrichment of EVs from a complex blood sample where they may be a minority presence, potentially using a panel of tissue-specific markers for enrichment, facilitates very focussed analysis of the signal that would otherwise be overwhelmed by more dominant and heterogeneous competition.

The challenges of using EVs for early cancer diagnostics

Despite the great potential of blood borne EVs for development of early diagnostics in cancer, there remain significant challenges. The first is related to their small size. The most commonly researched EVs, ‘exosomes’ at 30-150nm, are approximately the same size as a virus. This in itself poses issues for their collection, purification, quantification and handling. Moreover, there are obvious barriers to the collection of sufficient material for robust analysis, even prior to the issues discussed previously around enriching samples to achieve workable thresholds of sensitivity and specificity required for early detection. The challenges of working with EVs are well articulated in the literature, as exemplified by the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 [13], a position statement of the International Society for Extracellular Vesicles, which includes recommended protocols for their handling and analysis. The small sampling blood volumes that can be collected then require an increase in assay sensitivity to identify the EV analytes, which would exaggerate these issues. These and other preanalytical variables have led to the formation of the International Society for Extracellular Vesicles Rigor and Standardization Subcommittee to provide guidance to the community on these issues [59].

It is known that dietary lipoproteins [60], exercise [61], and pathologies other than cancer can also increase the level and change the content of EVs in the blood [62]. Circulating platelets can also significantly contribute to the EV landscape in blood, depending on their activation state [63,64], and they can release EVs if activated during sample processing. To reduce platelet contamination during the latter, there is a need for more understanding, and standardisation of sampling, including considerations of venepuncture methodology, centrifugation steps, freezing and storage conditions, which is progressing in the field [65,66] , and potentially a preference for the use of plasma over serum [67]. A further technical issue, which is especially critical when considering samples with very low signal intensity at early diagnosis, is the optimum medium for sample preparation and storage, such that biomarker integrity is retained prior to analysis. There is evidence that EVs in plasma are stable for up to 10 days when stored at 4ºC, and for up to 90 days when stored at -80ºC [68]. The importance of appropriate buffer formulation for storing EVs long term whilst retaining function, and the perceived potential market for EV research and applications, is exemplified by the filing of a 2019 patent on buffer composition for EV storage [69], but little is found in the current scientific literature. Furthermore, issues around appropriate long-term storage of samples for EV isolation and analysis are relevant to the wider infrastructure for research into biomarker discovery. For example, optimum storage of blood or serum samples in blood banks with EV applications in mind would open a significant resource to this field. Even allowing for this, the majority of blood samples collected by biobanks are currently from late, rather than early stage cancer patients and the search for novel early diagnostics may require thought given to the collection of blood from apparently healthy or asymptomatic individuals with subsequent follow up. An example of this is the UK Biobank initiative to store bloods from apparently healthy but aging individuals that are then followed over time to monitor subsequent emergence of clinically detectable disease [70].

By definition, the goal of development of an early diagnostic test is to detect a biomarker produced by a very small number of cells and its quantification at a minimum signal threshold in comparison to a normal baseline. In many cases, any significant downstream profiling analysis, such as proteomics or sequencing, will require significant amounts of sample. The issue of the small size of EVs is that their individual cargo is sparse. For example, Chevillet *et al* demonstrated that there is less than one molecule of any given microRNA per EV [71]. However, in mitigation, and as discussed previously, studies suggest that total EVs are highly abundant in blood. A systematic review collated 59 estimates of blood EV concentrations for healthy individuals over a range of extraction techniques and quantification techniques show a common value of around 10^{10} EVs per milliliter [72], the miRNA would only need to be present at 1 every 10,000,000 EVs in order for it to be detectable using qPCR [73]. It is important to mention that quantification of EVs is an important factor in diagnostics and often an issue of much debate around the potential clinical application of EVs. The limitations and specifications of quantification tools and technologies often focus on a specific EV characteristic. It can be challenging to be certain that the correct particles are being counted in a hugely diverse population of EVs, with contaminants seen in biological samples often adding to the quantification, as discussed by Rupert *et al* [74]. Maas *et al* reported technical issues and differences in absolute EV number when analysed by three commonly used technologies, nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (tRPS) and high-resolution flow cytometry (hFC) [75]. An early study by Rabinowits *et al* focused on EV number in early and late-stage lung cancer, but partly circumvented the issue by measuring the concentration of total protein and microRNA as a proxy for EV concentration [19]. This can be a controversial approach as challenges of clean EV extraction are problematic with contaminants changing total protein or RNA and therefore skewing the

counts. Even now, a decade later than this study, this approach of total EV counts would be considered technically challenging, requiring stringent sample preparation, with a reference control and beyond what could be regularly achievable in a routine clinical situation. Currently, there is interest in technical developments in EV quantification methods, and approaches are constantly improving, but there is still no single accepted technology to quantify EVs, and most studies use a combination of methods, or rely on sample comparisons depending on the circumstances [13].

In seeking to quantify changes in EV number or composition related to disease development, there are a number of pre-analytical factors that need to be overcome in order to provide a 'normal reference range', especially with the level of sensitivity that is required. These are well reviewed more generally for EV detection and profiling from blood [66], but it is worth focusing on some of the key elements required for increased sensitivity. The development of reference materials (both certified reference, quality control and calibrant materials [76]) are important for in-house assay design and calibrating machines across clinics. Natural reference materials for EV studies have been reviewed and the use of nanoerythrosomes (EVs released from erythrocytes) have been proposed, due to their similar refractive index to general EVs for flow cytometry analysis, larger diameter, surface CD235a to manipulate and the same lipids for labelling [77]. Hendrix and her group showed how engineered recombinant EVs can be used as a stable biological reference material, giving a more uniform but similar biochemical and biophysical characteristics in comparison to EVs extracted from biological samples. These recombinant EVs contain gag-EGFP fusion protein and EGFP mRNA and can therefore be added to samples, potentially providing a qualitative control [78]. Therefore, reference materials, quality controls and internal comparisons are important and widely discussed [79]. This is especially pertinent because, as described previously, one advantage of EVs over other blood-borne biomarkers such as CTCs is the potential for storage and later analysis. This is a critical issue going forward, as even when the quantification problems are solved, storage of blood samples across widespread clinical practice means that strict quality control is necessary because biomarker levels are likely to be at the lower end of the detection range [80].

The limited sample size can also limit the application of downstream technologies. A good strategy may be to use molecular tools based on amplification techniques, such as qPCR. Modern sequencing libraries only require between 100-1000 transcripts for a reliable signal, with some suggestion that qPCR could need as little as 16 molecules [73]. However, many of these technologies are semi-quantitative, which also reinforces the need for reliable reference samples. Digital PCR may help in this, as it allows absolute quantification of copy number, but to achieve meaningful quantification it will still require a validated housekeeping gene, and this is a challenge given the heterogeneity of EV composition.

Therefore, much of the focus for exosome diagnostics is on qualitative analysis of contents of the EVs, rather than their quantification. For clinically relevant biomarkers, the signal should be unique to the pathology and reliable within the chosen sample type. With any blood sample, there will be billions of EVs per ml, but it is unclear what proportion of these EVs are derived from a tumour (particularly for an early-stage tumour), and the ability to detect the signal will depend on the sensitivity of the assay employed to detect it. A recent computational EV kinetics model suggests that the current available bulk EV detection methods are around 10^4-fold too insensitive for the detection of a tumour sized 1 cm^3, but this detection is within reach of emerging single EV methods[81], as shown in the new section this is now reachable. There are many confounding factors that affect the signal to noise ratio when detecting the biomarker [59]. All cells release EVs, making them highly heterogeneous in

the blood. Evidence suggests each EV is probably unique, representing the molecular landscape of its parental cell. Many groups have reported EVs with different composition derived from the same cell type, and this heterogeneity may be exacerbated by slight changes in methods and size selection [82], as there is a suggestion that the internal protein cargo changes composition depending on the sampling and analysis methods [83].

Novel extracellular vesicle technologies and methods for early cancer diagnosis

There are several technologies that are rising to meet the challenges around sensitivity and background noise. This does not include highly sensitive research equipment for single-vesicle analysis, such as microscopy, that has challenges for clinical translation [84]. Many of these technologies either focus on nucleotide analysis by droplet digital PCR, as previously mentioned, or using the previous knowledge that common EV surface proteins, such as CD63 or CD81, or markers specific to target cells, such as EpCam on epithelial cells, to enrich the sample from the noise of the patient sample and then apply novel areas of engineering or analytical precision to show co-localised biomarkers. There is potential that this type of approach can become more sophisticated, once organ-specific markers have been reliably identified. Once the EVs have been enriched in the sample, the challenge of their small size and paucity of material remains, so a number of technologies are being developed to amplify their signal. Sina *et al* isolated breast cancer- derived EVs using HER2 on their surface, and then using surface plasmon resonance to detect as few as 2.07×10^3 to 3.3×10^4 EVs per microlitre [85]. Rojalin *et al* use a hybrid of cysteamine treatment on a metal surface to attract the EVs to surface-enhanced Raman scattering (SERS) as a rapid and inexpensive way to assay ovarian cancer EVs [86]. Microfluidics is another approach that can be used to both collect and enrich specific EVs from a range of fluids, and then focus them towards an analytic technology, a growing area reviewed by Lu *et al* [87]. Reátegui *et al* used a sensitive microfluidic platform with a detection limit of 100 EVs per microlitre to detect glioblastoma-derived EVs from patient blood samples [88]. Another team showed they could isolate prostate cancer-related EVs from 2ml of plasma taken from cancer patients by using antibodies directed against prostate specific antigen (PSA) on magnetic beads [89]. More recently, this sensitivity has been increased using sensitive Single Molecule Array (SIMOA) ELISA, for detection of EpCAM-positive EVs along with surface PD-L1, a clinically interesting cancer marker of immune system evasion, from as little as 250 µl of plasma [58]. There are now a range of single EV particle analysis detection methods available commercially that will be potentially vying for clinical access, including Nanoview's single-particle interferometric reflectance imaging sensing (SP-IRIS) [90], Particle-Metrix's nanoparticle tracking analysis (NTA) [25], Nanoparticle Analyser's microfluidic resistive pulse sensing (MRPS) [91] and NanoFCM's Nanoflow Cytometry Measurement (NFCM) [92]. A recent paper compares and discusses these platforms and shows that all have different advantages and limitations dependent on what is required of the analysis [93].

Recommendations for the development of EV early diagnostics in cancer

As discussed throughout this review, there is much progress to be made before the potential of measuring EVs in blood biopsies to diagnose cancer at an earlier stage can be realised. In summary, the key areas that need to be addressed are:

1. **Overcome the challenges of low levels of material** - Early detection of cancer-related biomarkers from EVs released into the bloodstream will rely on the technology providing resolution at a low level of signal. This is exacerbated by the paucity of EV cargo in a challenging background of competing signals. Hence, it is key that EV extraction, enrichment and detection are all refined. There are an increasing number of studies scrutinising different EV extraction methods [12,94], but the focus of these is often on gaining the purest samples for scientific exploration. There would be benefit in a clearer focus on how to translate such findings to a clinical setting that has different specifications and requirements. Much work on EV isolation has focused on smaller vesicles while larger EVs are often discarded during extraction, yet these could provide useful clinical biomarkers, and with their larger size have the capacity to carry more signal and be easier to manipulate. As an example, Vagner *et al* show that despite smaller EVs being greater in number than larger EVs in the plasma of prostate cancer patients, the larger EVs contained far more DNA with aberrations in common cancer-related genes [95]. A better understanding of the EV enrichment process would help drive the tuning for clinical applications. We recommend more work to be done on the selective enrichment or detection of EVs using tissue or organ-specific cargo that could enrich the signal. The EV field could learn from other '-omic' technologies, such as single-cell sequencing, that with large investment have effectively dealt with extraction, enrichment and signal amplification to gain reliable signals that bring new insights. Ultimately, the issue of low levels of EV cargo is purely a technical one, which requires advances in EV methodology. Standards or reference materials, mentioned earlier will help drive this technological tuning. Indeed, the potential commercial gains have led several companies to develop technologies with increased sensitivity and specificity alongside a strategy to address the clinical market. Even with these improving platforms, there are numerous hurdles before new technology can enter the complex clinical healthcare landscape, but overcoming these initial barriers is critical for realising the potential of EVs in early cancer diagnostics.

2. **Address and risk-assess the pre-analytical variables and heterogeneity in EVs**- Changes in EV composition can be seen in several physiological states, including during pregnancy [96], exercise [61] and during changes to diet [60]. Blood EVs are affected by methods of collection, processing, extraction and storage [13]. Taken together, it is clear that there is still a great deal more work that needs to be undertaken to understand these potential variables and to apply the knowledge to EV extraction and analysis in routine clinical practice. One area that EV clinical science would benefit hugely from, would be to understand any influences that biobanking methods may have on the EVs. Biobanks are an EV biomarker 'goldmine' for the study of many diseases, including cancer, and if we understood the confounding factors that biobanking methods may have on samples, we may be able to compensate for them in the subsequent analysis. This requires better standardisation in the methods of EV extraction, handling, storage and downstream analysis, such as that exemplified by the International Society of Extracellular Vesicles task forces, comprising teams of scientists that are generating frameworks and guidelines [12]. This needs to be expanded to include broader dialogue from clinical teams, especially those in oncology, pathology, analytics and nursing.

3. **Profile changes in EV cargo through all stages of cancer development -** There is great heterogeneity in analytical profiles between individual cells within a tumour mass, which will be reflected in the EVs that these individual cancer cells release, and importantly this will also change over disease progression from early to late stage disease, especially in the different characteristic transitions seen over different tumour types. There is therefore a need for approaches to profile and identify EV cargo molecules that are reflective of the stages in cancer development, from early premalignant or latent disease, early detection of primary cancer, through to detection of metastases. This is a particularly pertinent issue since there is evidence that EVs themselves can participate in tumour progression by preparing the tumour microenvironment [18]. Inherent EV heterogeneity may require the use of combined panels of different molecule types to be used in diagnosis and prognostication to differentiate different stages of disease progression. There are several sensitive and advanced technologies for 3D and spatial genomics that could help identify, extract and characterise specific EVs in tumour microenvironments, exemplified in The Human Cell Atlas [97] linked to microscopic guided cell and tissue extraction. These could potentially also support the profiling of EVs at different stages and roles in cancer development. Although they are often expensive and analytically very demanding, these types of approaches are likely to become more widely used over time. Biopsy profiling in patients and *in-vivo* models to identify specific EV subsets related to stages of tumour development could facilitate earlier diagnosis, and determination of prognosis, before clinically detectable disease is apparent using current imaging technologies. We therefore recommend the continued acquisition of EV profiling data from increasing numbers of patients at different stages of disease.

4. **Increase access to pre-symptomatic clinical samples -** Obtaining clinical samples from patients at an early stage in their disease is a challenging task, especially if the goal for early detection is to identify pre-symptomatic patients. This is particularly true of cancers like ovarian cancer that often remain undiagnosed until at an advanced stage. Thus, there needs to be a strategy of working with clinicians on the collection of early-stage samples, and provision of access to samples from those at highest risk of developing cancer because of advancing age or familial history. This could be extended to screening programmes and wider clinical trials and studies. Projects like the UK Biobank [70] provide a hugely valuable resource for potential EV profiling of blood taken from 500,000 'healthy' participants with detailed follow up, many of whom have since developed cancer. These samples are precious, so a clear and robust approach to their analysis needs to be established before they are utilised, but would bring huge benefits in mapping cancer progression.

5. **Launch an extracellular vesicle 'moonshot' -** There needs to be a wide-scale and ambitious project with a cross-disciplinary focus that brings insights and expertise from disciplines including engineering, physics, chemistry, and materials science. The importance of this is demonstrated by the integrated extraction microfluidics and sensitive clinical testing technologies that have already seen some pre-market success [88]. Such an effort could include smaller academic groups, and be coordinated by one or more centres of excellence. The success of projects such as the 100,000 Genome Project [98] and The Cancer Genome Project [99] have revolutionised how we stratify treatments for individual patients. In these instances for general cancer research, the

move from analysis of small sample groups to an ambitious population level genomic, proteomic, epigenomic, and lipidomic approach, with greater bioinformatic power and whole genome level information, has overcome some of the heterogeneity issues seen between individual tumour samples and provided the raw data for thousands of researchers. As far as EV research is concerned, one approach might be to combine with projects like these, adding the EV analytics to the blood already taken to compare with clinical data and adding an additional dimension to the existing data. This is also true of aligning data with cfDNA and CTC tools. The stakeholders previously mentioned along with health economists, healthcare technology providers and regulators supported by government, industry and charities need to guide current research so that either novel single-molecule platforms can develop faster for the clinical market, or academic assay developers integrate healthcare needs into their designs for better bench-to-bedside translation.

Conclusion

There are some true benefits for the potential role of EVs in the early diagnostics of cancer, but this comes with its challenges as EV science is still in its early stages. The development of more reliable EV extraction methods and analytical platforms, the potential adaptation of other genomic technologies and understanding, the clinical shift to healthcare becoming more personalised, and the promise of early clinical studies has led to a real interest in this space. Much of this science is ready for clinical translation, and with the right stakeholders and support, this could potentially revolutionise early cancer diagnostics. Characteristics of EVs such as their stability and their mirroring of their parental cell in terms of composition, plus the technical capability to extract low levels of signal from background noise, makes them an intriguing proposition for use in blood biopsies. They therefore have the potential to shift healthcare from the present reactive state to a more proactive system.

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