Targets, pitfalls and reference materials for liquid biopsy tests in cancer diagnostics

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1. Introduction

Cancer is a genetic disease arising from molecular alterations in genes involved in cell survival, growth, proliferation and differentiation making cells escape the regulatory cell cycle and form their own ecosystem (tumor) that can intervene with the physiology of organs, resulting in organ failure and death if not treated properly (Hanahan and Weinberg, 2011). Cancer can be treated by general cell death-inducing agents, which are commonly accompanied with severe side effects, reducing the patients' comfort of life. Targeted therapies intervene with molecular pathways triggered in cancer cells by genetic alterations (DeVita and Chu, 2008; Schirrmacher, 2019). Currently, the genetic characterization of tumors to obtain information for therapy selection and prognosis prediction is based on tissue biopsies of the primary tumor itself or metastases. However, tumors are a heterogeneous agglomeration of cell subpopulations each carrying a distinct set of molecular alterations. Additionally, different metastatic lesions at different locations in the body can have a different molecular profile. This can result in the procurement of therapy resistance, since only a fraction of the tumor or metastatic sites will be targeted, while other subpopulations, carrying other mutations, can thrive and advance cancer progression (Dagogo-Jack and Shaw, 2018; McGranahan and Swanton, 2017). To cope with this heterogeneity, scientists took advantage of the rapid turnover of cancer cells resulting in an increased release of cancer associated materials in their extracellular environment. Biomarkers contain relevant information concerning normal or abnormal physiological processes at the single cell level. Biomarkers originating from tumors (cancer biomarkers), including circulating tumor cells (CTC), proteins, cell free DNA (cfDNA) and RNA (cfRNA) and extracellular vesicles (EV), can represent the molecular status of the heterogeneous tumor or metastases since these materials are released from different sites in the tumor or metastatic lesions (Giulia et al., 2017; Murtaza et al., 2015). Biomarkers can be released directly into bodily fluids or indirectly due to disrupted tissues. The assessment of biomarkers in biological fluids, termed "liquid biopsy", has gained increasing interest, especially for cancer, because of the relative ease of obtaining genetic information without the need of performing invasive surgery. Besides non-invasive sampling, liquid biopsies bring forward new possibilities for cancer diagnosis and care: treatment response of a certain therapy can be easily monitored (Annala et al., 2018; Murtaza et al., 2015; Pantel and Alix-Panabières, 2019) and screening of population groups at risk by simple blood sampling could increase the early detection of cancer and hereby increase the survival rate of patients (Babayan and Pantel, 2018; Cohen et al., 2018). Cancer biomarkers can be sampled from bodily fluids such as blood, urine, saliva, cerebrospinal fluid, stool and lavage fluids. In this review we will focus on blood-based liquid biopsies.

Although research on circulating cancer biomarkers has resulted in a plethora of possible targets, little progress has been made towards the clinical application of liquid biopsy tests because of the lack of clinical
A reference material, according to ISO guide 30:2015, is “a material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process”. Important notes that accompany this definition are that: 1) the term “reference material” is a generic term; 2) the properties can be quantitative or qualitative; 3) the uses can include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control; and 4) it can only be used for a single purpose in a given measurement.

The generic term “reference material” can further be subdivided into: 1) certified reference materials, which are characterised by a meteorologically valid procedure for one or more specified properties, property, its associated uncertainty, and a statement of metrological reference materials often produced in-house, thus are not sufficiently characterised to provide metrological traceability; and 2) calibrants, which should have a fixed property value with an appropriate uncertainty tolerable for calibration and metrological traceability of the property value. In theory, a calibrant should always be a certified reference material, but in practice they often lack proper certification; however, a certified reference material is not always a calibrant because the uncertainty stated in the certificate is not always tolerable for calibration (Emons, 2006). Since there are very few certified reference materials available for liquid biopsy tests, in this review we will use the terms pre-analytical reference material for materials being used for calibration, quality control and assessment of measurement methods, and analytical reference materials for materials being used to assign values to other materials or defining the LOD of measurement methods. The former can be used to assess pre-analytical variables and their influence on experiments, while the latter can be used for liquid biopsy testing, by comparing its property value with that of the patient material (Fig. 1).

Commutability of a reference material implies that it behaves comparably to the actual sample undergoing the procedure and as such is a procedure-specific characteristic, defined by the behaviour of the reference material (Vesper et al., 2007). The correct assessment of reference material commutability is important, as biases will arise when using non-commutable reference materials, leading to inaccurate results. A reference material can be spiked in samples (spike-in) and used to assess and mitigate technical errors between samples by functioning as an internal quantitative or qualitative control, or can be used as a scaling factor for normalization purposes between samples. Alternatively, a reference material can be used as an external control to compare obtained results or to assign specific property values to a sample for diagnosis. In general, commutability is harder to achieve for a spike-in compared to an external control. Additionally, a spike-in reference material should be distinguishable from the molecules under investigation (Hardwick et al., 2017).

validity and utility (Watts, 2018). In order to achieve clinical validity and utility of liquid biopsy tests, its analytical validity must be assessed, followed by prospective studies using the protocols which resulted in analytical validity. Analytical validity includes the accuracy, sensitivity, specificity and robustness of the liquid biopsy test and is dependent on pre-analytical variables and protocols used for sample preparation and biomarker detection (Merker et al., 2018). Pre-analytical variables are factors without direct disease association that impact the integrity of the bodily fluid or biomarker present in that bodily fluid, or influence results during analysis and can be of a technical, biological or environmental origin. The impact of pre-analytical variables is dependent on the biomarker and bodily fluid studied and should be thoroughly assessed for each newly developed liquid biopsy test (Agrawal et al., 2018; Ellervik and Vaught, 2015). Standard operating procedures (SOP) for the complete workflow of liquid biopsy tests are crucial to advance their clinical implementation (Freedman and Inglese, 2014; Khleif et al., 2010; Merker et al., 2018). For the assessment of analytical validity and the influence of pre-analytical variables as well as for the optimization of SOP, reference materials with known properties are invaluable. Reference materials (see box 1), can further be used to detect experimental failure, to calibrate and assess the lower limit of detection (LOD) of measurement methods, and to assist in biomarker quantitation.

Here, we review the technical considerations, pre-analytical variables and reference materials of the most commonly studied targets for the development of liquid biopsy tests for cancer, including cfDNA, CTC, EV and cfRNA.

2. The assessment of cfDNA in liquid biopsies

The presence of fragmented DNA in the non-cellular component of blood, also termed cell free DNA (cfDNA), was first described in 1948 (Mandel and Metais, 1948). The importance of cfDNA was only recognized in 1994 when a mutated RAS gene fragment was detected in the blood plasma of pancreatic cancer patients (Sorenson et al., 1994). cfDNA is thought to be released from cells during apoptosis and necrosis, and possibly also through active secretion (Stroun et al., 2001; Thakur et al., 2014). Enzymatic cleavage of DNA during apoptosis results in the formation of DNA fragments of on average 166 bp, corresponding to DNA wrapped around a single nucleosome. Larger fragments starting from 320 bp, the length of DNA wrapped around two nucleosomes, up to > 1000 bp are released from phagocytotic or necrotic cells (Lo et al., 2010; Thierry et al., 2010). Concentrations of cfDNA in blood plasma have been reported to range from 1.8 to 44 ng/ml with a half-life shorter than 2.5 h (Fleischhacker and Schmidt, 2007; Sorenson et al., 1994; Tie et al., 2015). The hematopoietic system, particularly the white blood cells, is the predominant source of cfDNA (Schwarzenbach et al., 2011; Snyder et al., 2016). cfDNA that is released from cancer cells, is also referred to as circulating tumor DNA (ctDNA). In cancer patients, shorter ctDNA fragments compared to normal cfDNA fragments in the range of 90–150 bp are detected in blood plasma (Cristiano et al., 2019; Mouliere et al., 2018). The concentration of ctDNA in blood plasma varies among cancer patients depending on type, location and stage of cancer and is frequently low (Bettegowda et al., 2014).

The detection of somatic mutations, commonly single base-pair alterations, copy number variations (CNV) or chromosomal rearrangements in ctDNA shows promise for early cancer diagnosis, tumor dynamics assessment, minimal residual disease (MRD) detection and therapy monitoring. Mutations identified in ctDNA extracted from blood plasma and in matched tumor tissue of cancer patients show a high concordance rate, encouraging routine implementation of ctDNA testing as an adjunct to tumor testing (Rothwell et al., 2019). Detection of cancer-specific mutations in ctDNA following resection of breast and colorectal tumors has been shown to identify patients destined to relapse post-operatively in advance of established clinical parameters (Beaver et al., 2014; Garcia-Murillas et al., 2015; Tie et al., 2016). ctDNA also carries tissue and cancer specific epigenetic aberrations and methylation profiles of tumor tissue highly correlate with those from ctDNA (Xu et al., 2017). In 2016 the US Food and Drug Administration (FDA) approved the first two blood-based assays (cobas EGFR Mutation Test v2, Roche Molecular Systems and Epi proColon, Epigenomics) for the detection of respectively mutations in the EGFR gene in non-small cell lung cancer and methylated SEPT9 gene in colon cancer.
2.1. Technological considerations

Screening for clinically relevant mutations in ctDNA is challenging because ctDNA is highly fragmented and because it is masked by a high background of total cfDNA, resulting in allele frequencies (AF) lower than 0.1%, especially for early stage tumors or micrometastases (Bettegowda et al., 2014; Li et al., 2019). Although significant progress has been made in advancing the detection and analysis of ctDNA in the last few years, the current challenges include standardization and improving current methods to reach single molecule sensitivity in combination with high specificity.

Blood contains whole blood cells. During sample processing, storage and transport, cell lysis may occur resulting in the release of genomic DNA (gDNA) that contaminates the cfDNA fraction and potentially lead to false negative results. There are different strategies to assess the degree of gDNA contamination. DNA capillary electrophoresis allows to estimate the DNA fragment sizes. Quantification of short and long sequences of the same gene using quantitative PCR (qPCR) enables the analysis of the amplicon ratio long/short. To prevent gDNA contamination, specialized blood tubes are available that stabilize blood cells and prevent disruption (Johansson et al., 2019; Nikolaev et al., 2018; Norton et al., 2013).

Different methods are commercially available to extract cfDNA from blood plasma (Fong et al., 2009; Trigg et al., 2018). An optimal cfDNA extraction approach should purify all cfDNA fragments to the same extent, maximize yield and minimize the presence of PCR inhibitors. Magnetic enrichment of cfDNA is achieved by binding with functionalized magnetic beads, whereas silica column-based enrichment makes use of the binding affinity of DNA molecules at specific buffer conditions. cfDNA can also be captured by polymer mediated precipitation, in which the cfDNA is not soluble, or by a phenol-chloroform based extraction (Ali et al., 2017). Today, most methods are based on either magnetic beads or silica-based membranes.

ctDNA can be detected by enrichment using PCR, digital PCR or next generation sequencing (NGS) (Li et al., 2019). The former two are targeted approaches based on the analysis of a single or a selection of
clinically actionable mutations. This approach requires a priori knowledge of the target mutation but has a high sensitivity and specificity and is fast and cost-effective. NGS-based detection relies on untargeted screening of the extracted cfDNA for the presence of clinically actionable mutations. A priori knowledge of the mutation is not required for this approach but NGS introduces errors during amplification, library preparation and sequencing, resulting in error rates of 0.1–0.5%, making it impossible to detect ctDNA with AF lower than 0.5%. Recent approaches to increase the sensitivity of NGS, such as Safe-SeqS, CAPP-Seq and TAmSeq have succeeded and require high level expertise or specific bioinformatic approaches (Newman et al., 2016; Pécuchet et al., 2016; Schmitt et al., 2012). Another approach to enhance NGS sensitivity is by increasing the bodily fluid input volume, hereby increasing the total amount of cfDNA (Johansson et al., 2019). Further developments to improve the detection of ctDNA with NGS-based screening include selecting specific sizes of cfDNA fragments prior to sequencing, assessment of the epigenetic methylome, development of bioinformatics algorithms and improving standardization (Cristiano et al., 2019; Mouliere et al., 2018; Xu et al., 2017; Zavridou et al., 2018).

2.2. Pre-analytical variables

Interlaboratory variation can be induced by pre-analytical variables such as extraction and quantification method used, storage conditions, PCR inhibition and fragment size bias (Fleischhacker et al., 2011; Fleischhacker and Schmidt, 2007; Khleif et al., 2010; Trigg et al., 2018; van Dessel et al., 2017; Whale et al., 2018). To date no consensus SOP is available to assess cfDNA in blood plasma, but research concerning the performance of cfDNA extraction, synthetic or exogenous DNA sequences of different length can be spiked in known concentrations in blood plasma prior to extraction. By comparing the quantitative signal after extraction with the signal of the reference material that was not spiked, the extraction efficiency can be assessed, as well as fragment size bias of the extraction (Whale et al., 2018). The ADH plasmid, which shows no homology with the human genome, treated with restriction enzymes resulting in DNA fragments of 115, 461 and 1448 bp is commonly used for this purpose, however other exogenous DNA sequences can also be used. Using this fragmented plasmid, Devonshire et al. compared different cfDNA extraction kits and observed that certain kits extract more efficiently shorter fragments, while others are biased towards larger fragments (Devonshire et al., 2014).

Spiking synthetic or exogenous DNA sequences after cfDNA extraction helps to identify the presence of PCR inhibitors (Whale et al., 2018). PCR inhibitors can be introduced during blood sample collection, cfDNA extraction or over-concentrating the extracted cfDNA. The most commonly known PCR inhibitors are heparin, hormones and IgG, which lower the efficiency of the PCR reaction resulting in higher Cq values or lower read counts for qPCR or NGS respectively (Johansson et al., 2019; Sah et al., 2013). Higher Cq values or lower read counts for the spiked reference material compared to the pure reference material are indicative for the presence of PCR inhibitors (Devonshire et al., 2014; Johansson et al., 2019; Whale et al., 2018). PCR inhibition can be resolved by either diluting or re-extracting the cfDNA.

2.3. Reference materials for cfDNA

2.3.1. Pre-analytical reference materials

Reference materials for the assessment of pre-analytical variables in cfDNA research should only match the physical characteristics of cfDNA, of which the fragment size is the most important one. To assess the performance of cfDNA extraction, synthetic or exogenous DNA sequences of different length can be spiked in known concentrations in blood plasma prior to extraction. By comparing the quantitative signal after extraction with the signal of the reference material that was not spiked, the extraction efficiency can be assessed, as well as fragment size bias of the extraction (Whale et al., 2018). The ADH plasmid, which shows no homology with the human genome, treated with restriction enzymes resulting in DNA fragments of 115, 461 and 1448 bp is commonly used for this purpose, however other exogenous DNA sequences can also be used. Using this fragmented plasmid, Devonshire et al. compared different cfDNA extraction kits and observed that certain kits extract more efficiently shorter fragments, while others are biased towards larger fragments (Devonshire et al., 2014).

Further developments to improve the detection of ctDNA with NGS-based screening include selecting specific sizes of cfDNA fragments prior to sequencing, assessment of the epigenetic methylome, development of bioinformatics algorithms and improving standardization (Cristiano et al., 2019; Mouliere et al., 2018; Xu et al., 2017; Zavridou et al., 2018).

Plasma is preferred to serum for the extraction of cfDNA, as the latter is contaminated with gDNA due to blood cell lysis that occurs during clotting (Li et al., 2019; Merker et al., 2018). EDTA tubes can be used as long as they are processed in less than 6 h at room temperature, or 24 h when stored at 4°C. After these time points an increase of gDNA is observed. Alternatively, specialized tubes that prevent cell lysis can be used (PAxgene tubes, Qiagen; BCT tubes, Streck Inc.; cfDNA collection tubes, Roche Diagnostics), which allow the storage of blood plasma up to 48 h at room temperature prior to processing (Grölz et al., 2018; Merker et al., 2018; Nikolaev et al., 2018; van Dessel et al., 2017). Two centrifugation steps above 800 g and longer than 10 min are preferred to one in order to avoid contamination of the plasma with white blood cells. The centrifugation speed or temperature does not impact cfDNA recovery (Trigg et al., 2018). Storage of processed plasma samples at ~80°C up to one year does not degrade cfDNA. Longer storage at ~80°C results in 30% degradation per year. Multiple freeze thawing steps of blood plasma and freeze thawing of whole blood is not recommended (El Messaoudi et al., 2013; Merker et al., 2018; Sozzi et al., 2005).

A multitude of cfDNA extraction methods are available. Comparative studies of different cfDNA extraction methods revealed method dependent cfDNA yields, fragment lengths and detection of target genes (Devonshire et al., 2014; Fleischhacker et al., 2011). Awareness of pre-analytical variables and their impact is important and requires transparent reporting to increase interlaboratory reproducibility.

2.3. Reference materials for cfDNA

2.3.2. Analytical reference materials

Analytical reference materials should not only match the physical characteristics but also the biological characteristics of ctDNA. For the detection of ctDNA, especially with NGS, one should be able to detect and distinguish somatic mutations from germline cfDNA. It is thus important for analytical reference materials to contain a matched germline cfDNA background together with a minor fraction of ctDNA. The most ideal reference material would be cfDNA from clinically validated patients. However, since cfDNA is only present in limited amounts in bodily fluids and reference materials should have little variation between batches, this is not practically feasible.

For the evaluation of liquid biopsy tests based on ctDNA, the LOD should be thoroughly assessed to exclude false negative results. The LOD can be assessed by introducing a specific mutation or alteration in gDNA and mixing this mutated gDNA with control background gDNA at specified AF. However, genome editing is prone to off target effects and PCR-based mutagenesis can introduce random errors, making this a less reliable reference material (Yang et al., 2017). A much considered approach to assess the LOD is to isolate DNA from well characterised cell lines, mostly transformed cells from patient samples carrying clinical variants of interest, fragment this DNA by ultrasonication and mix the DNA fragments with isogenic control DNA at fixed concentrations to reach pre-set AF. With these mixes, each containing ctDNA at decreasing concentrations, the LOD can be estimated (Denroche et al., 2015; Harkins et al., 2016; Zhang et al., 2017). Nonetheless, ultrasonication results in relatively broad size distributions and damaged DNA ends, hereby adversely affecting the commutability of the reference material during isolation and analysis (Konigshofer et al., 2017; “Seraseq ctDNA: A Breakthrough QC Technology,”; Zhang et al., 2017).

Zhang and colleagues mimicked the nucleosomal footprint of cfDNA by treating CRISPR/Cas9 edited cells, bearing frequently occurring genomic rearrangements, with micrococcal nuclease (MNase). The latter is able to digest DNA between nucleosomes in vivo (Tsang and Chan, 2017; Zhang et al., 2017). This ctDNA mimic can then be mixed with MNase treated control DNA to achieve pre-set AF. In their publication they propose a synthetic cfDNA quality control material (SQQC) existing from MNase treated CRISPR/Cas9 edited and control cells and control DNA that underwent PCR-based site directed mutagenesis to introduce 6 frequently occurring driver mutations. They state that this SQQC can be used for the monitoring of false-positive and false-negative results. A major limitation of this reference material is...
the lack of cfDNA extraction from complex bodily fluids. Also, it is impractical to spike this reference material in blood, as it already contains cfDNA, not matching the background of the reference material. Ideally, a reference material should be applicable for the evaluation of the complete cfDNA analysis workflow (from plasma preparation to analysis). To meet this need, NIST, in collaboration with SeraCare, has developed cfDNA reference materials for multiple disorders at pre-defined AF in a synthetic human plasma matrix (Königshofer et al., 2017; “Seraseq ctDNA: A Breakthrough QC Technology,”).

Such analytical reference materials are not restricted to assess the LOD but are also applicable for the quantitative assessment of ctDNA in patient samples. By preparing reference materials with increasing AF, a standard curve can be constructed from which the AF of the ctDNA sequence of interest can be deduced (Denroche et al., 2015; Hardwick et al., 2017). This is particularly of interest for therapy response monitoring and MRD assessment, where the ctDNA AF is correlated with the tumor load.

3. The assessment of CTC in liquid biopsies

The first documented case of CTC dates back from 1869, where Thomas Ashworth observed cells in the blood of a man, deceased due to metastatic disease, that resembled the primary tumor cells microscopically (Ashworth, 1869). During both late and early stages primary tumors shed cells in the circulation that can seed at distant sites of the body and form metastatic lesions (Braun et al., 2005; Massagué and Obenauf, 2016). CTC are a rare event, with 1 CTC present for 1 × 10⁸ – 1 × 10⁹ blood cells depending on the disease state (Tibbe et al., 2007). They have a half-life of 1–2.4h and can be distinguished from mesenchymal blood cells by the expression of epithelial surface proteins, such as epithelial cell adhesion molecule (EpCam) or proteins of the cytokeratin family (CK8, CK18 and CK19), or by their epithelial morphology (Alix-Panabières and Pantel, 2013; Giulia et al., 2017; Lianidou and Markou, 2011; Meng et al., 2004; Pantel et al., 2012). However, cancer cells in the circulation can undergo epithelial to mesenchymal transition (EMT), losing their epithelial phenotype and surpassing the immune system with a mesenchymal phenotype. Along this process, the expression of cytokeratins and EpCam is lost and the morphology is altered (Alix-Panabières et al., 2017; Yu et al., 2013). Some studies report that these mesenchymal CTC are more prone to form metastases (Bourcy et al., 2016; Bulfon et al., 2016; Mego et al., 2012).

CTC can be captured from the circulation, quantified and characterised to aid molecular identification of the primary tumor or metastases, and therapy response monitoring (Lianidou and Pantel, 2019). Detection of cancer-specific splice variants by assessing mRNA of CTC identifies patients destined to develop therapy resistance (Antonarakis et al., 2014; Armstrong et al., 2019). Patients with a higher CTC count have a more than two fold less median overall survival compared to patients with a lower CTC count (Cristofanilli et al., 2019). To date the CellSearch system, which is based on enumeration, is the only CTC-based FDA-approved test (Riethdorf et al., 2018, 2007).

3.1. Technological considerations

CTC have to be enriched from blood which already contains millions of cells. Characterization and enumeration of CTC is typically not performed on pure CTC isolates but on 3–4 log enriched CTC, aiming at the presence of 1 CTC for 1000 blood cells or less. Since CTC follow the Poisson distribution, the LOD is defined by the amount of blood that is available (Tibbe et al., 2007). This is reflected by the necessity of 22.5 mL or 7.5 mL of blood to detect CTC with high fidelity in early or metastasized cancer patients respectively, making use of the CellSearch system (Lianidou and Pantel, 2015; Riethdorf et al., 2018). This relatively large amount of blood can be overcome by in vivo CTC enrichment by inserting a nanodevice via a standard gauge needle in the bloodstream of a patient for 30 min, hereby increasing the chance of effectively capturing a CTC (Alix-Panabières and Pantel, 2013; Saucedo-Zeni et al., 2012).

CTC can be enriched from the blood via positive or negative selection based on protein expression or physical properties. Enrichment based on protein expression, the most implemented method, makes use of specific proteins present on the surface of CTC that are absent on the surface of circulating blood cells in case of positive selection or the other way around in case of negative selection. Commonly used targets for these enrichment strategies comprise the epithelial surface protein EpCam or blood cell specific surface proteins such as CD45. Enrichment based on physical properties makes use of the size, density, deformability or electric charges specific for CTC (Alix-Panabières and Pantel, 2014; Kho et al., 2017; Rawal et al., 2017).

Detection, enumeration and characterization of CTC is mostly performed concomitantly with the enrichment in a microfluidic device. This can be done by using fluorescent imaging, molecular assays (mostly PCR-based) or protein assays (detecting tumor specific proteins released by CTC). The number and characteristics of CTC can be assessed by distinguishing CTC from contaminating blood cells with Immunocytochemistry (ICC), fluorescent in situ hybridisation (FISH) or padlock probe assays together with DAPI staining; PCR-based methods for molecular characterization are sensitive enough to detect CTC-associated signatures in these conditions (Alix-Panabières and Pantel, 2014; El-Heliebi et al., 2018; Lianidou and Markou, 2011).

The restricted use of epithelial cell markers for CTC enrichment poses problems for the specificity and sensitivity of the assay. First of all, CTC that underwent EMT lose their epithelial-specific proteins and physical properties. This implies that they are not captured by classical CTC isolation methods, resulting in an increase of the false negative detection rate (Alix-Panabières et al., 2017; Yu et al., 2013). Therefore, it should be considered to not only focus on epithelial markers, but also on mesenchymal markers without increasing the false positive rate by identifying blood cells as CTC. To date, there is no consensus on potential mesenchymal targets that could be used, however, the use of surface expressed vimentin could pose a solution (Satelli et al., 2015). Furthermore, a combination of mesenchymal capturing strategies and FISH assays for the nuclear organisation of transcription factors, like transforming growth factor (TGF) β-1 or FOXI1, or molecular assays to identify tumor specific alterations are also under consideration (Wu et al., 2016; Yu et al., 2013). Another argument that could stand against the use of EpCam to capture CTC is the fact that there are reports of circulating epithelial cells in patients with benign diseases, which could result in false positive results (Pantel et al., 2012). Also, different sub-populations of CTC exist which are more prone to extravasation and metastasis formation than others. These CTC are characterised by a specific set of surface proteins including CD47, CD44, MET and EpCam but also HER2, EGFR, HSPE and notch (Baccelli et al., 2013; Zhang et al., 2013).

To obtain information about intra-patient heterogeneity, genome analysis should be performed on individual CTC. Single cell sequencing makes use of whole genome amplification, which is prone to systematic errors that can result into false findings. Investment in single cell analysis methods is thus highly needed for accurate diagnosis making in the future (Alix-Panabières and Pantel, 2014).

3.2. Pre-analytical variables

The most important, and most analysed, pre-analytical variables that can impact downstream analysis of CTC are the type of blood collection tube used, time between sampling and analysis and storage temperature of whole blood. Depending on the downstream method of analysis, pre-analytical variables may have different impacts.

Blood collection aimed at CTC analysis should be performed in EDTA containing blood collection tubes or tubes designed for cfDNA isolation containing a formaldehyde free preservative to ensure the
structurally stable CTC. However, the choice of either EDTA or preservative containing tubes can have a major impact on the obtained results (Grölz et al., 2018). For enumeration and characterization based on imaging, ICC, FISH or padlock probe analysis directly after blood draw (less than 6 h) no consensus is available on which blood tube to use (Flores et al., 2010; Luk et al., 2017). When the time to analysis is increased, preservative containing tubes are preferred (Ilie et al., 2018). However, caution is advised since these tubes were initially developed for cfDNA research, where they were used to minimize gDNA contamination of lysed leukocytes, hence leukocytes present in the blood are also preserved and could interfere with the analysis (Luk et al., 2017). For molecular characterization of CTC, through PCR-based approaches, preservative containing tubes are strongly discouraged. Even when CTC enrichment and analysis are performed directly after sampling, significantly low DNA or RNA yields were obtained, after more than 24 h almost no nucleic acids were found when using preservative containing tubes. Using EDTA tubes, CTC associated nucleic acids could still be detected up to 96 h later (Luk et al., 2017; Wong et al., 2017; Zavridou et al., 2018). If the aim is to culture CTC ex-vivo or the generation of a xenograft model, preservatives cannot be used as these contain fixatives that interfere with the cell cycle.

Blood samples for CTC analysis can be stored at room temperature without significant effects up to 72 h (Apostolou et al., 2017; Grölz et al., 2018). For molecular characterization however, one could reason that storage at room temperature could influence RT-qPCR results as a consequence of up- or downregulation of transcripts as an active response to cellular stress (Benoy et al., 2006). Therefore, storage of EDTA tube collected blood for molecular characterization of CTC could better be performed at 4 °C. This again poses a problem for CTC enrichment, which is mostly performed in microfluidic devices, as hypothermic temperatures result in platelet activation with clotting as a result, which could lead to failure of the microfluidic device. Wong et al. have bypassed this problem by the addition of glycoprotein IIb/IIIa inhibitors to EDTA blood collection tubes, which resulted in stable live CTC isolation for up to 72 h with high quality RNA for molecular characterization (Wong et al., 2017).

Another important variable, not a pre-analytical but an analytical variable, which should be appreciated is inter-reader variability. Since CTC are rare events, severely outnumbered by hematological cells, different operators could obtain different cut-off values. Standardization of the interpretation of CTC analysis tests, through specialists training, is thus highly recommended (Cummings et al., 2013).

3.3. Reference materials for CTC

3.3.1. Pre-analytical reference materials

All pre-analytical variables discussed above were assessed by using established in vitro cultures from specific cancer types. Known concentrations of cancer cells, cultured in vitro, are spiked in whole blood and subsequently the recovery is assessed under varying test conditions. Obtained results should always be validated using patient material because these cells are adapted for in-vitro culture and have undergone structural and molecular changes (Lipps et al., 2013). Especially for the assessment and validation of CTC isolation methods this poses a serious concern. It has been observed that CTC can significantly differ from their cell culture counter parts on the basis of size, morphology, nucleocytoplasmic ratio and fluorescence intensity after staining for certain markers (Lazar et al., 2012; Park et al., 2014). The best possible reference material to assess these variables correctly is by using CTC isolated from patients, but since these cells are so rare, it is impossible to provide sufficient material for wide spread use. This could be overcome by the immortalization of isolated CTC for in vitro culture. However, immortalization can impact signalling pathways and only few attempts in long time culture of CTC have succeeded (Franken et al., 2019; Maheswaran and Haber, 2015; Pantel and Alix-Panabières, 2015; Yu et al., 2018).

3.3.2. Analytical reference materials

An optimal analytical reference material should be able to help define the MRD in a patient, by assessing CTC count or the AF of altered genes when looking at remaining CTC after therapy. Such a reference material already exists for chronic myelogenous leukaemia and Philadelphia chromosome-positive acute lymphoblastic leukaemia (White et al., 2010). This reference material consists of mixtures of freeze dried K562 (BCR-ABL positive) and HL60 (BCR-ABL negative) cells at fixed 10%, 1%, 0.1% and 0.01% MRD, which is meant for the calibration and certification of secondary reference materials. By using this reference material, or secondary materials calibrated with it, clinicians can with high reproducibility and certainty deduce the MRD of a patient after therapy.

A first approach of developing such an analytical reference material was published recently. Tommasi and colleagues implemented prostate cancer LnCap cells, carrying known mutations of the androgen receptor (AR), in three different concentrations as a reference material to standardize RT-qPCR analysis, assess the LOD and evaluate other pre-analytical variables (Tommasi et al., 2019).

Further development of similar reference materials is hampered by the absence of consensus on using molecular analysis of CTC as a prognostic or predictive biomarker. Optimization of single cell analysis methods is thus first needed before these kind of analytical reference materials would find use in a clinical setting.

4. Assessment of extracellular vesicles in liquid biopsies

Extracellular vesicles (EV) are nanovesicles ranging from 50 to 1,000 nm secreted by all cell types and consist of a lipid bilayer surrounding a cargo consisting of proteins, nucleic acids and metabolites (Kalra et al., 2016; van Niel et al., 2018). EV can bud directly from the plasma membrane or can be released by fusion of a multivesicular body (late endosome) with the plasma membrane, giving rise to respectively microvesicles or exosomes. Apoptotic bodies, arising as membrane blebs during the apoptotic process, are also categorized as EV. Each EV subpopulation has its own route of biogenesis and can be characterised by or share a specific set of proteins. However, up until now there is no consensus on the classification of EV subtypes based on protein characteristics (Baetti et al., 2012; Jepessen et al., 2019; Kowal et al., 2016; Nabhan et al., 2012; Tulkens et al., 2018; Zhang et al., 2018). EV are involved in intercellular communication both locally and at greater distances, making use of the blood- or lymph circulation (Maas et al., 2017; Valadi et al., 2007; Zomer et al., 2015). Half-lives have been reported in mice ranging from 20 to 180 min and the EV concentration in healthy individuals is approximately 1.46 × 10⁹/mL plasma (Geeurickx et al., 2019; Lai et al., 2014).

EV are secreted by cancer cells and can either activate or recruit cancer or stromal cells locally or educate a premetastatic niche at a distant location, hereby facilitating metastasis formation (Hoshino et al., 2015; Kalluri, 2016; Peinado et al., 2012; Tkach and Théry, 2016). Recently, EV separated from different bodily fluids have been found to contain specific mRNA, miRNA and protein content related to the disease state for multiple cancer types. This makes them interesting targets for liquid biopsies as they represent a fingerprint of the originating cell (Melo et al., 2015; Nawaz et al., 2014; Sadowska et al., 2015; Tang and Wong, 2015; Zhang et al., 2015). Not only their cargo but also their numbers in plasma can be indicative for cancer and recurrence after therapy (Osti et al., 2019).

Research into extracellular vesicles (EV) raises hope to gain biological insights and identify novel diagnostics and therapeutics for a wide range of pathological conditions. However, the plethora of methods to separate and characterize EV, the intrinsic heterogeneity of EV subtypes and the complexity of bodily fluids block the road towards rigor in EV research and clinical application (De Wever and Hendrix, 2019).
**4.1. Technological considerations**

EV are most commonly separated from bodily fluids by differential ultracentrifugation (dUC), size exclusion chromatography (SEC), density gradient (DG) centrifugation and immune capture targeting EV-associated tetrascansins CD9, CD63 and CD81, but commercial precipitation methods also exist (Coomans et al., 2017a; Théry et al., 2018; van der Pol et al., 2016). More than 1000 unique EV separation methods have been reported in literature, each separating EV with different specificity and efficiency (Van Deun et al., 2017, 2014). To understand the functional significance of intercellular communication via EV, the EV-specific proteome, transcriptome and lipidome should be accurately defined without the presence of non-vesicular contaminants such as abundant proteins or lipoproteins present in blood. The employment of a density gradient is up until now the only method to discern EV from protein complexes and should be used to validate the association of a biomarker with EV (Théry et al., 2018; Van Deun et al., 2017). However, a density gradient cannot discern EV from high density lipoproteins abundantly present in blood and able to bind miRNA. As such, a combination of multiple methods (size and density) can help to obtain higher specificity (Karimi et al., 2018; Onodi et al., 2018; Simonsen, 2017; Tulkens et al., 2018). The International Society for Extracellular Vesicles (ISEV) has recently defined the minimal experimental requirements to identify an EV-associated biomarker (Théry et al., 2018).

Although the EV concentration in blood is increased in cancer patients (Geurickx et al., 2019; Ost et al., 2019), the majority of circulating EV is released by hematopoietic cells. Since most EV separation methods are based on generic biophysical or biochemical properties of EV, tumor-specific EV cannot be distinguished from hematopoietic EV. This problem could be circumvented by direct capture and analysis in unprocessed or partly processed bodily fluids by targeting cancer-specific membrane proteins, which should also be present in the membrane of their respective EV (Melo et al., 2015; Zhang et al., 2019). Sizing and quantification of EV is mostly based on their light scattering properties through nanoparticle tracking analysis (NTA) and flow cytometry (FCM), although EM or resistive pulse sensing are occasionally performed for these purposes as well. Each method has its limitations and merits, and it is important to take them into account during analysis. Light scattering methods lack the ability to properly distinguish vesicles from non-vesicular particles, thus these particles could be mistakenly quantified as EV (Filipe et al., 2016; Van Deun et al., 2014). The amount of light that is scattered by a certain particle of a certain size is related to the refractive index, which for EV is low. This consequently defines the LOD, which is dependent on the sensitivity of the camera used by the specific method (Dragovic et al., 2011; van der Pol et al., 2014). For this reason it is impossible to compare the measured concentration from one method to another without the use of a calibrator (Valkonen et al., 2017; van der Pol et al., 2014). The low refractive index also restricts the minimum size of EV that can be assessed, larger particles scatter more light with the result that smaller particles cannot be assessed under the same conditions. Alternatively, fluorescently labelled antibodies targeting EV-associated tetrascansins and FCM could be used (Kormelink et al., 2015). Using this methodology, results could be biased as there is a possibility that only a subpopulation of EV is labelled. Another phenomenon that can cause misinterpretation of results is the smear effect, where multiple EV pass the laser at the same time resulting in one count. This can be corrected for by performing multiple dilutions of the same sample and re-measuring it until a linear correlation is obtained (Kormelink et al., 2015; van der Pol et al., 2012). EM has the advantage that the operator can choose what is quantified, giving the possibility to not include protein aggregates in the analysis. Recently a tool was developed to standardize vesicle sizing and counting, making this technique more approachable (Kotrbová et al., 2019). However, during EM samples are centrifuged, fixed and dehydrated resulting in loss and morphological changes of EV, again influencing the analysis (van der Pol et al., 2014). Methods to analyse EV for biomarker discovery are dependent on the EV-associated molecule being studied. Since EV can contain cancer cell related RNA, DNA, proteins or lipids the analysis methods range from RT-qPCR, NGS, proteomics and lipidomics being performed concomitantly with (on a microfluidic device) or after EV separation (Coomans et al., 2017a; Gézsi et al., 2019; van der Pol et al., 2016).

**4.2. Pre-analytical variables**

The EV community is fully aware of the complexity of analysing EV in bodily fluids and therefore ISEV published several position papers concerning the above-mentioned pitfalls and the influence of pre-analytical variables (Mateescu et al., 2017; Théry et al., 2018; Witwer et al., 2013).

When isolating EV from blood, plasma is the preferred matrix compared to serum because platelets secrete an increasing amount of EV during coagulation which could result up to 50% of the total amount of EV present in serum (Coomans et al., 2017a; Gemmell and SeftonYeo, 1993). When preparing plasma, the preferred anti-coagulant is sodium citrate and platelets should be depleted, preferably by double centrifugation for 15min at 2500g. Plasma should be prepared in a maximum time span of 2h and can be frozen at −20°C and −80°C up to one year without impact on EV size and concentration (Vuana et al., 2015). However, when separated, EV should be frozen at −80°C up to one year without impact on EV size and concentration (Vuana et al., 2015). Important patient-associated pre-analytical factors when working with blood are prandial status, level of exercise and inflammation, as these are known to alter EV levels in patient plasma (György et al., 2011). When storing, concentrating or measuring EV protein concentration it is also advised to consider the materials to use as it has been noticed that some vials and cut-off membrane tend to be more sticky to EV and that some protein concentration methods over- or underestimate the real protein concentration (Vergauwen et al., 2017). Important patient-associated pre-analytical factors when working with blood are prandial status, level of exercise and inflammation, as these are known to alter EV levels in patient plasma (György et al., 2011; Witwer et al., 2013).

The plethora of methods and protocols can be confusing, but it is probably unavoidable, given the diverse nature of EV and innovative character of the EV research field (De Weyer and Hendrix, 2019). To cope with this variety of methods the scientific community needs to increase the awareness of essential experimental parameters and needs to report them sufficiently to make EV research transparent, so we can understand each other’s experiments and reproduce data. Specifically, the EV-TRACK knowledgebase (http://evtrack.org/) is an online open access resource to track and organize data on EV separation and characterization and therefore to monitor the progress in the field of EV in a standardized format. In the current set-up, a check-list of nine essential experimental parameters to improve the transparency in the field are identified and bundled into an EV-METRIC (Van Deun et al., 2017). Guidance to use EV-TRACK and its EV-METRIC is previously reported (Van Deun and Hendrix, 2017).

**4.3. Reference materials for EV**

Light scattering methods to detect EV require proper calibration to ensure that the correct size and concentration is actually measured. For these purposes polystyrene or silica beads with pre-set size and concentrations are often used. However the refractive indices of these
beads are respectively 1.61 and 1.46 compared to a refractive index of approximately 1.39 that is reported for EV (Gardiner et al., 2013; van der Pol et al., 2014; van der Pol et al., 2018). Since the refractive index of a certain material correlates with the amount of light that is scattered, these beads scatter more light than EV. When calibrating a system with beads, comparable bead sizes as EV will be used but due to their higher refractive index, they will scatter as much light as 2–3 fold larger EV. This will result in missing the smaller population of EV and consequently underestimating the true EV concentration (Chandler et al., 2011). Recently, Varga and colleagues proposed hollow organosilica beads (HOB) as a suitable alternative (Varga et al., 2018). They produced monodisperse 200 nm and 400 nm beads based on silica with a similar refractive index as EV by mimicking the high scattering properties of the EV membrane and the low scattering properties of the EV lumen. They used these HOB to set nanometer size gates for EV quantification with FCM. However, since these HOB are still monodisperse, while EV are heterogeneous in size, and cannot yet be produced in 100 nm size ranges, the size mode of circulating EV, optimization is still encouraged. It has been shown for instance that measurements with FCM, NTA and TRPS resulted in larger discrepancies in quantification for monodisperse liposomes compared to EV preparations (Maaß et al., 2015).

While HOB possess a favourable refractive index, they lack all other relevant EV properties, limiting their use solely to the calibration of light scattering technologies. Since the characterization of EV is not limited to light scattering methods alone, but also protein-, RNA-, lipid- and image-based methods are being used for EV characterization, it would be convenient for a reference material to contain other relevant EV properties. According to researchers working with EV, biochemical composition similar to EV and stability are the two most appreciated properties of an EV reference material (Valkonen et al., 2017). Liposomes could be proposed as they are fully customizable to achieve any EV-like properties: RNA and proteins can be incorporated, the lipid composition can be varied and the density and refractive index can be modulated. Liposomes have been used for the calculation of recovery efficiencies of EV separation methods by spiking a known concentration (based on total lipid concentration) in serum free cell culture medium and after EV separation measuring the remaining lipid concentration (Lane et al., 2015). Although this system gives a good representation of the performance of EV separation methods, it cannot be used for recovery determination in bodily fluids as these already contain lipids prior to the spike-in. Liposomes have also been shown to be able to express the large extracellular loop of EV-associated tetraspansins in their membrane (Lozano-Andrés et al., 2019). These EV-mimetics can be used as a positive control for fluorescence triggering high resolution FCM and the calibration of fluorescent signals after staining with a membrane dye or with fluorescent antibodies targeting the large extracellular loop of CD9, CD63 or CD81. More complex lipid vesicles can also be generated from whole cells. Ultrasonication of erythrocytes results in vesicles ranging from 200 to 400 nm in size with the same morphology, density and refractive index as erythrocyte EV, these vesicles are termed NanoE (Valkonen et al., 2017). The protein composition of NanoE is different from EV and they do not contain significant amounts of nucleic acids so they cannot be used as positive control in protein or nucleic acid enrichment experiments, but they have been shown to be able to function as an inter-measurement calibrator for NTA and FCM.

Possibly the most appropriate reference material for EV are actual EV from cell cultures or bodily fluids, or derivatives thereof, as these contain multiple EV properties. Recently, Görgens et al. proposed EV, containing a fusion protein of CD63 and enhanced green fluorescent protein (EGFP), as a biological reference material for EV analysis. They showed that these EV could be detected above the threshold with imaging FCM based on fluorescent triggering and that these EV could be used for the evaluation of acquisition parameters of the instrument and EV labelling experiments. Additionally, these CD63-EGFP positive EV can also be used to assess loss of EV during EV separation from cell culture medium (Görgens et al., 2019). For the same purposes as the CD63-EGFP EV, fluorescently labelled murine leukaemia viruses (MLV) have also been proposed. Additionally, these MLV based reference materials can be modulated to express any surface protein, making them interesting to evaluate the labelling of cancer specific surface markers (Chatterjee et al., 2011; Tang et al., 2019). Finally, we recently proposed recombinant EV (rEV) as a biological reference material for quality control, data normalization, method development and calibration of EV measurement methods. rEV were intensively characterised and found representative of sample EV and commutable during EV separation from bodily fluids. rEV are based on a fusion protein of the HIV-1 major structural component gag and EGFP, which renders them highly fluorescent and rich in EGFP mRNA. The fusion protein allows to track rEV during separation making use of fluorescence-, protein- or RNA based methods, by which they can be used to normalize quantitative results of bodily fluid-derived EV (Geeurickx et al., 2019).

5. Assessment of cfRNA in liquid biopsies

Compared to the analysis of somatic mutations in gDNA, which only provides information about the molecular condition of the cell of origin, the analysis of transcribed RNA species can give complementary information concerning gene expression profiles or epigenetic alterations. This is especially important for therapy response monitoring in cancer patients, as therapy resistance often relies on epigenetic changes (Esteller, 2011). In 1996 the presence of RNA in the blood of a cancer patient was first reported, it concerned tyrosinase mRNA and its concentration correlated with the stage of melanoma (Stevens et al., 1996). Following this finding, publications linking the presence of RNA in blood to cancer remained modest up until the finding of stable microRNA (miRNA) species present in the blood of prostate cancer patients (Mitchell et al., 2008). Nowadays, cell free miRNAs (cRNA) are viewed as the most important RNA species for biomarker discovery because of their higher abundance, tumor- and tissue-specific profile, stability and their potential to alter gene expression levels upon single stimuli (Giulia et al., 2017; Lu et al., 2005). The stability of miRNAs in RNAse-rich blood is due to complexation with proteins, lipoproteins or platelets, or to incorporation in EV, which can protect against RNAse digestion; this is not seen for circulating mRNA, limiting their potential as circulating cancer biomarkers (Arroyo et al., 2011; Joosse and Pantel, 2015; Valadi et al., 2007; Vickers et al., 2011). The exact mechanism of extracellular RNA release is not yet known for non-vesicle associated cRNA but, as with cDNA, release during apoptosis or necrosis is hypothesized.

The hematopoietic system is also the predominant source of cRNA. Hematopoietic cells can release the same miRNAs as cancer cells, hereby hampering the identification of a true cancer biomarker (Pritchard et al., 2012). The biological function of the same miRNA released by a cancer cell or a hematopoietic cell however is not necessarily the same because epigenetic regulation through miRNAs is a complex process which can occur by a single miRNA or several miRNAs working cooperatively (Calin and Croce, 2006; Esteller, 2011). Consequently, when looking for a true miRNA-based cancer biomarker it is better to search for miRNA profiles than to focus on a single miRNA as has been shown to be useful for hepatocellular carcinoma and breast cancer patients diagnosis (Montani et al., 2015; Tan et al., 2019). Potential cRNA as cancer biomarker are not limited to originating from cancer cells themselves, also cells in the tumor microenvironment release miRNAs which can promote tumor development and are thus important for diagnosis making (Okada et al., 2010).

Since miRNAs are the most studied population of cRNA as potential cancer biomarkers, we will mainly focus on this subtype when discussing the technological considerations, pre-analytical variables and reference materials.
5.1. Technological considerations

RNA can be isolated from bodily fluids using commercial isolation kits based on phenol/guanidinium extraction, affinity columns or a combination of the two. When focussing on miRNA, an additional size exclusion step through electrophoresis can be included (Schwarzenbach et al., 2014). Since miRNAs are associated to different compartments in the blood (protein, lipoproteins, platelets and EV), each requiring different isolation procedures (centrifugation speeds, size gates and immune-affinity), the subset of isolated and extracted miRNA will be dependent on the used protocol. As there is no evidence present which subset is of prior importance for biomarker discovery it could be interesting to analyse the whole blood compartment or at least the whole plasma/serum compartment for cfmiRNAs (McDonald et al., 2011).

Because miRNAs are particularly small (18–25 nt) and have specialized secondary structures, they are not only difficult to extract but also adaptations had to be made to conventional RT-qPCR and NGS analysis methods for increased detection (Buschmann et al., 2016; Kroh et al., 2010; Schwarzenbach et al., 2014; T'Hoen et al., 2013). When analysing cfmiRNAs, the input sample will always be a mixture of small RNAs (under which miRNAs) and large RNAs because RNA extraction methods lack specificity to only extract miRNAs. As such, concentration measurement of miRNAs is cumbersome and will always be over-estimated by the presence of longer RNAs. Therefore, it is more convenient to work with fixed volumes when comparing different samples (Kroh et al., 2010; Ono et al., 2015). Capillary electrophoresis after RNA extraction allows to exclusively analyse miRNA, but caution is advised as it is impossible to distinguish precursor and mature miRNA.

Cells present in blood contain high concentrations of miRNA which can potentially influence the detection of cancer specific miRNA profiles. It is thus of high importance to deplete the bodily fluid completely of whole cells and concomitantly prevent them from lysing before analysing cfmiRNA. Similar blood collection tubes as used for cfDNA analysis can be used for the purposes of cfmiRNA analysis. The first drawn blood tube should be discarded, as this can contain epidermal cell contamination by disruption of the skin with the needle (Ono et al., 2015).

5.2. Pre-analytical variables

Due to the novelty of the cfmiRNA field no consensus exists on how to properly treat bodily fluids and perform miRNA analysis. The influence of pre-analytical variables is being studied at a steady pace, but not as extensively as in the cfDNA, CTC and EV field yet.

cfmiRNA can be isolated from both plasma and serum, no real preference is set for either one of them. Although differences were found in the miRNA profiles of both kind of bodily fluids, the total cfRNA concentration remained similar (Kroh et al., 2010; McDonald et al., 2011; Ono et al., 2015). When isolating cfRNA from plasma or serum, the inflammation state of the patient and the complete blood cell count should be monitored. This because the majority of proposed single miRNA-based cancer biomarkers have been found to be attributed to the white blood cell count and type, so potentially only pointing out a secondary effect of leukocytes rather than cancer cells (Pritchard et al., 2012). Additionally, hemolysis also affects the miRNA profile in blood by increased release of the content of red blood cells after dis-ruption. Hemolysis should not only be checked visually but also spectrosocopically by absorbance measurements at 414 nm. Absorbance values lower than 0.2 at this wavelength do not result in miRNA fluctuations in the same sample (Kirschner et al., 2011). The nutritional state of patients can also drastically impact the profiles of miRNAs in blood, probably by an increased lipoprotein concentration, to which miRNAs can bind to (Marzi et al., 2016; Vickers et al., 2011).

RNA extraction is a major contributor of irreproducibility, multiple studies have conducted comparisons of different commercial RNA isolation kits for the analysis of cfmiRNA and most point out that the miRNAseq (Qiagen) results in the highest yields (Marzi et al., 2016). RNA extraction has also been found to be variable between different input volumes as no correlation was found with the input volume and reported Cq values. This is due to saturation of the column by an excess of proteins (Androvic et al., 2019). Due to its small size and low concentration, miRNAs can be lost during extraction by sticking to the plastic. This can be prevented by addition of carrier tRNA or glycogen, the latter has been found to be better because an excess of tRNA can potentially influence the analysis (Androvic et al., 2019). Variability during RNA extraction can be reduced by working with fixed volumes, not only the input volumes, but also when separating the aqueous phase when using phenol/guanidinium-based extractions (Marzi et al., 2016).

Expression values of miRNAs in plasma and serum were found stable after multiple freeze thaw cycles at ~ 80 °C, although storage at 4 °C for up to 72 h did not seem to impact the expression profiles (Kroh et al., 2010).

RNA expression is subjected to multiple stimuli and can therefore easily vary between samples, even when there is no causality. Therefore, miRNA profile normalization should be performed with good internal reference genes, preferably found to be stable in all conditions of the experiment (Bustin et al., 2009). Up until now, no good reference controls have been found but miR-16, miR-30b and miR-142-3p are often used as internal normalizers, although the first one has been found to be influenced by hemolysis (Ono et al., 2015; Pritchard et al., 2012; Schaefer et al., 2010). Another way of normalizing for miRNA expression is the use of mean miRNA expression values, which has been found to be relatively invariable throughout multiple samples. When looking for appropriate reference genes, the stability thereof should be compared to the mean miRNA expression value (Mestdagh et al., 2009).

When using the same input samples, reproducibility, specificity, sensitivity and accuracy can still vary between miRNA expression platforms, even when these platforms are based on the same technologies. qPCR-based platforms for instance have been found to have a higher sensitivity and accuracy when it comes to low input samples. This suggests that the miRNA expression platform should be chosen based on the experimental settings (Mestdagh et al., 2014).

5.3. Reference materials for cfRNA

To partially correct for errors during extraction, an often-used method is to spike non-homologous miRNA molecules, for instance C. elegans miRNAs, in the sample undergoing extraction. This spike-in should not be performed in the untreated bodily fluid, since they often contain a high concentration of RNases, but after adding denaturation buffers. It is especially important that these spike-ins contain 5’ terminal phosphate to allow them to be incorporated into miRNA libraries for NGS. These non-homologous miRNA sequences can also be spiked after extraction and prior to reverse transcription and PCR to check for the presence of PCR inhibitors, as explained above for cfDNA reference materials. Regarding this, an effort to standardize the miRNA workflow was recently published where they made use of 3 extraction spike-ins, 2 RT spike-ins and 3 endogenous miRNA to assess the performance of miRNA extraction, the presence of PCR inhibitors and the contamination of cellular miRNA originating from erythrocytes (Androvic et al., 2019).

The field of cfRNA is still in its infancys and RNA expression analysis methods, especially sequencing methods, are still being optimized. To optimize these methods, well characterised reference materials are strongly needed that mimic true RNA species in physiological concentration ranges. To address this need, the External RNA Control Consortium (ERCC) have put in remarkable effort to develop a spike-in control to optimize the RNA expression field (Baker et al., 2005; Cronin et al., 2004). This ERCC reference material consist of 96 polyadenylated transcripts that mimic natural eukaryotic miRNAs but are not identical (less than 0.01% of reads mapped to the human genome). They are...
designed to have a wide range of lengths (250–2000 nucleotides) and GC-contents (5–51%) and can be spiked into RNA samples before library preparation at various concentrations to assess the RNA sequencing workflow. Standard curves can be achieved with the ERCC spikes, demonstrating a linear quantification over 6 orders of magnitude, proving that they can also be used to normalize RNA sequencing data (Jiang et al., 2011). However, caution must be given when using these ERCC spikes for normalization since it was demonstrated that the read counts are affected by the biological factors studied (Risso et al., 2014).

More recently, a related spike-in material was developed to assess the small-RNA sequencing workflow. Locati and colleagues developed two different spike-in sets. Size-range quality controls (SRQC) set which can be used to assess size selection bias of NGS, as the library preparation makes use of size exclusion steps. SRQC consist of 11
synthetically synthesized, single-stranded 5′-phosphorylated RNA sequences of increasing length (10–70 nucleotides). External reference for data normalization (ERDN) set which consist of 19 single-stranded 5′-phosphorylated RNA sequences of 25 nucleotides that cover the physiological expression range of small RNAs in blood. This spike-in set can be used to normalize small RNA sequencing data based on the total input prior to analysis (Locati et al., 2015).

As there is still much to optimize in the RNA sequencing workflow, most of the RNA reference materials being developed are concerning this. Due to the limited scope of this review we will not go further into detail while providing further references for review (Buschmann et al., 2016; Hardwick et al., 2017, 2016; Munro et al., 2014; Paul et al., 2016; Risso et al., 2014). Because of this high need to standardize the RNA sequencing workflow, no efforts were conducted to develop analytical reference materials yet. The question remains however whether this will ever take place because miRNA profiles in a complex bodily fluid are difficult to mimic compared to single mutation detection or detection of mutated cells.

6. Measures for increased reproducibility in the liquid biopsy field

Liquid biopsies hold great potential for future diagnosis, prognosis and therapy monitoring of cancer patients. However, only three liquid biopsy tests are FDA approved, two ctDNA- (cobas EGFR Mutation Test v2 and Epi proColon) and a CTC-based test (CellSearch). The reason for this low FDA approval rate is the general low level of reproducibility in pre-clinical research. This lack of reproducibility is particularly higher for cancer research compared to other research fields, supposedly due to the higher urgency for finding solutions in oncology (Begley and Ellis, 2012). With a high concentration of researchers working on cancer detection through analysing hard-to-isolate biomarkers from complex bodily fluids, the field is over flooded with lab-specific protocols for isolation and analysis. This was illustrated by a meta-analysis of EV research where 1226 EV related articles from 2010 to 2015 were thoroughly analysed and found reporting 190 unique EV isolation methods, which resulted in 1038 unique protocols through adaptations of previous methods. This, together with incomplete reporting of the experimental set-up, hampers unambiguous interpretation or replication of experiments (Van Deun et al., 2017).

Since the increased attention for the lack of reproducibility in pre-clinical cancer research, the global biological standard institute (GBSI) published the case for standards in which they assessed the quality of research, identified areas of concern, and proposed recommendations for improvements (Freedman and Inglese, 2014; “The Case for Standards in Life Science Research: Seizing Opportunities at a Time of Critical Need,”). With this publication GBSI aimed at raising awareness for standards among all stakeholders of life science research and its progress, and stimulating them to engage in community efforts to create consensus standards in life science research. Standards imply both reference materials and consensus documents generated by professional communities. The latter can range from simple SOP to more complex standards for data analysis or accurate reporting guidelines. Such consensus documents are important for the correct execution of experiments, interpretation of results and for the findability of published results (Freedman et al., 2017). Such standards should not directly come from standards developing organisations (SDO), such as ISO (International Organization for Standardization) or CEN (European Committee for Standardization) (de jure standards) but should be proposed by community efforts after lengthy discussions with all stakeholders in the field, including researchers, publishers and funders (de facto standards). The most important community efforts for standardisation of the liquid biopsy field and their impact are summarized in Table 1. Important for the generation of community standards is to include as much stakeholders as possible and prevent the generation of multiple standards for the same topic. This can result in fragmentation of community efforts and prevent the implementation of real community standards in practice (Cargill, 2011; Sansone and Rocca-Serra, 2012). Recently, an informative and educational resource tool was launched, FAIRsharing, that describes and interlinks community standards, databases, repositories and data policies in one searchable database (Sansone et al., 2019). With this database researchers, funders and publishers can lookup resources to identify standards, databases or repositories that exist for their data and discipline with the goal to respectively improve the success rate of their experiments, increase the impact of the funded work and reduce irreproducible data being published by enforcing topic-specific standards.

Reference materials can also be developed by community efforts followed by commercialization. ctDNA reference materials provided by SeraCare and Horizon were developed in collaboration with respectively the National Institute for Standards and Technology (NIST) – Early Detection Research Network (EDRN) collaborative (NIST-ERDN) in the US and LGC limited in the UK. Such reference materials can be trusted to represent the subject of interest, as it is a result of broad community discussions, proficiency testing and external quality assessment (EQA) (Haselmann et al., 2018). However, reference materials can also be commercially provided by private companies without participation of community efforts. These reference materials should be tested appropriately because they are mostly not subjected to rigorous proficiency testing and EQA. At its best, a reference material must be fully traceable and the producer or reference material itself should be certified by an SDO. In the case of ISO these producers or materials should follow ISO 30:2015, ISO 31:2015, ISO 33:2015, ISO 35:2015, ISO 17034:2016 guides. However, traceability is difficult to achieve for biological reference materials. A reference material that adheres to all these criteria is available for chronic myelogenous leukaemia and Philadelphia chromosome-positive acute lymphoblastic leukaemia and is approved by the WHO (White et al., 2010).

7. Concluding remarks

Liquid biopsies hold much promise for improving early cancer detection and monitoring therapy response, consequently improving overall patient survival. However, liquid biopsy research projects rarely find their way to clinical application because of a lack of reproducibility (Merker et al., 2018; Watts, 2018). Community established SOP and reference materials should be developed to avoid the influence of pre-analytical variables and increase reproducibility of measurement methods. In the last decade, multiple community efforts focussing on the standardization of the liquid biopsy field were initiated and contributed as such. The rise of these community efforts could mean the turning point of irreproducible pre-clinical studies and hopefully the implementation of liquid biopsy tests in the clinic.

Author contributions

E.G. and A.H. co-wrote the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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