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Review Article

The pre-analytical phase of the liquid biopsy

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ABSTRACT

The term 'liquid biopsy', introduced in 2013 in reference to the analysis of circulating tumour cells (CTCs) in cancer patients, was extended to cell-free nucleic acids (cfNAs) circulating in blood and other body fluids. CTCs and cfNAs are now considered diagnostic and prognostic markers, used as surrogate materials for the molecular characterisation of solid tumours, in particular for research on tumour-specific or actionable somatic mutations. Molecular characterisation of cfNAs and CTCs (especially at the single cell level) is technically challenging, requiring highly sensitive and specific methods and/or multi-step processes. The analysis of the liquid biopsy relies on a plethora of methods whose standardisation cannot be accomplished without disclosing criticisms related to the pre-analytical phase. Thus, pre-analytical factors potentially influencing downstream cellular and molecular analyses must be considered in order to translate the liquid biopsy approach into clinical practice. The present review summarises the most recent reports in this field, discussing the main pre-analytical aspects related to CTCs, cfNAs and exosomes in blood samples for liquid biopsy analysis. A short discussion on non-blood liquid biopsy samples is also included.

Introduction

The NCI Dictionary of cancer terms defines 'liquid biopsy' (LB) as 'a test done on a sample of blood to look for cancer cells from a tumour that are circulating in the blood or for pieces of DNA from tumour cells that are in the blood' [1]. The term was first introduced in 2013 in reference to the analysis of circulating tumour cells (CTCs) in cancer patients [2], and was subsequently extended to cell-free nucleic acids (cfNAs) in the blood and other body fluids. Furthermore, cfNAs include circulating cell free DNA (cfDNA), exosomes, microRNAs (miRNAs), other noncoding RNAs (ncRNAs) and circulating gene transcripts (cfRNA) [3] released into the circulation by most cancers at any stage of disease. The study of the LB has opened the perspective of a non-invasive tumour diagnosis and characterisation, which has been applied to the early detection of cancer, the identification of therapeutic targets and the monitoring of the response to therapy for precision medicine, as well as the disclosure of mechanisms of disease progression [4].

The workflow of LB analysis is a complex combination of several steps, which have not yet reached complete standardisation and can vary depending on the biomarker under study. Moreover, the pre-analytical phase can differ considerably depending on the blood compartment under investigation. Fig. 1 shows a schematic description of the different procedures included under this term, differentiating cfNAs, CTCs and extracellular vesicles. The pre-examination process starts with the preliminary blood collection phase, which can require the stabilisation of the sample and includes storage and transport. This phase is common in all the components of the LB, while the subsequent phases involve workflows of differing complexity, based on the localisation of the biomarker. In fact, sample centrifugation to separate the liquid part of blood from blood cells and nucleic acid extraction will follow if the aim of the study is to analyse cfNAs; alternatively, an enrichment/isolation phase is needed if the object of the analysis is contained within tumour-derived cells or vesicles (for CTCs or exosomes) prior to a staining/imaging step or the extraction of biomolecules from the enriched preparations.

It is worth underlining that the pre-analytical phase for LB analysis

Abbreviations: LB, liquid biopsy; CTC, circulating tumour cell; cfDNA, cell-free DNA; cfRNA, cell-free RNA; cfNAs, cell free nucleic acids; miRNA, microRNA; ncRNA, non coding RNA; lncRNA, long non coding RNA; EQA, External Quality Assurance; EPCAM, epithelial cell adhesion molecule; CK, cytokeratin; NGS, next generation sequencing; WGA, whole genome amplification; WTA, whole transcriptome amplification; EV, extracellular vesicle Piwi-interacting RNA (piRNA); ctDNA, circulating tumour DNA; dPCR, digital PCR

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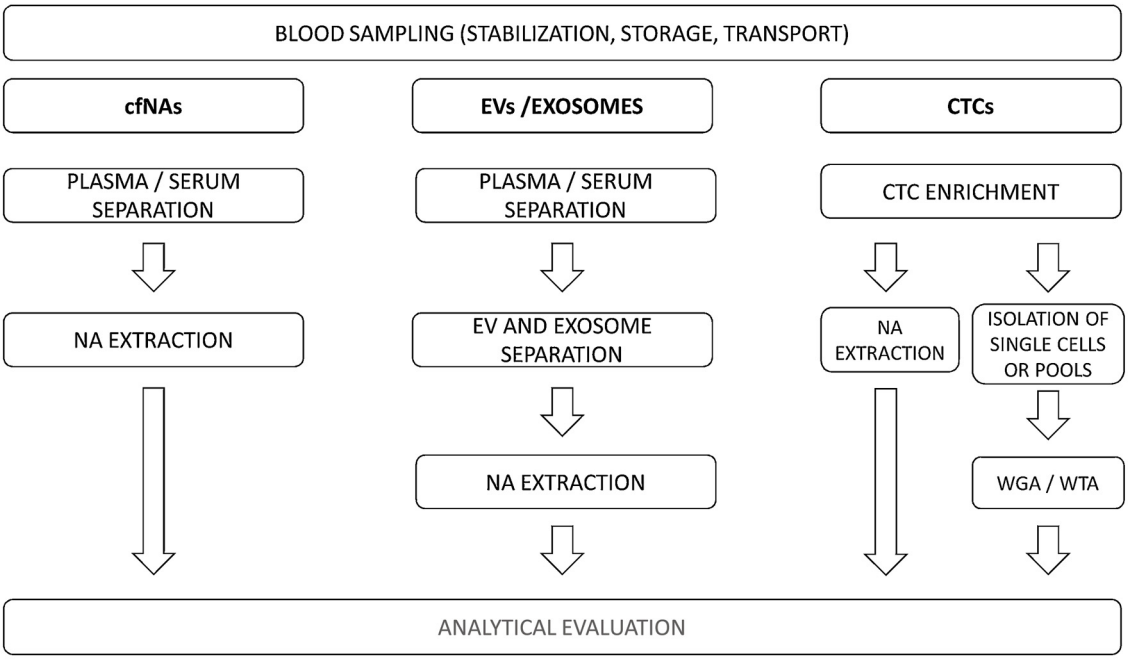

Fig. 1. Schematic representation of the pre-analytical phase for the different components of the Liquid Biopsy.

cannot be uniquely defined, and that the term refers to many different procedures, not only on the basis of the substrate of choice (plasma, tumour cells or vesicles) but also of the examination process to be performed. Consequently, an obvious but useful definition of the pre-analytical workflow can be formulated including all the phases preceding the analytical examination. Here, cfNAs are referred to as nucleic acids obtained from plasma or serum samples (with no enrichment phase), whereas nucleic acids associated with cells or vesicles will be considered as being obtained by an enrichment and/or an isolation step from blood or plasma, as is usually performed for investigation of nucleic acids in exosomes and CTCs.

In regard to CTCs, the main pre-analytical critical aspects are related to their fragility [5]; in fact, they tend to degrade within a few hours when collected in standard blood collection tubes (e.g. EDTA containing tubes). Moreover, CTCs are rare, especially in early stages of the disease, and are detected at a very low frequency among billions of blood cells, a background that contaminates sample purity and contributes to challenge the CTC enrichment phase. Further, standardised pre-analytical protocols are needed to ensure reliable and efficient methods for CTC detection as well as their molecular characterisation. Nucleic acids from CTCs can be either extracted or amplified upon direct lysis; in both cases, the pre-analytical phase has to be carefully standardised and validated. On the other hand, cfNAs are also characterised by a low and variable concentration in plasma or in serum and cfNA profiles can change significantly after blood collection (e.g. release of genomic DNA from cells in blood, cfNA fragmentation and degradation), due to which special measures need to be taken to obtain good quality and an appropriate quantity of nucleic acids for downstream analyses [6,7].

In terms of exosome studies, rapid advances in understanding their biological functions have been providing new insights into the roles that they play in the diagnosis, treatment and response to therapy in cancer [8,9]. Similar to CTC and cfNA analysis, exosome analysis outcome is strongly influenced by pre-analytical treatment [10–13]: exosomes have to be reliably and efficiently isolated from complex biological matrices like blood, urine and other body fluids; thus, standardised and consistent protocols for sample collection and preparation are needed.

Currently, there is no standardisation of the whole pre-analytical process for LB analysis even if some progress has been made, especially for blood sample stabilisation for cfDNA, cfRNA and CTC integrity. Nonetheless, most pre-analytical aspects still require deep investigation and harmonisation among different research laboratories, making it difficult to compare the obtained results. As part of the EU project SPIDIA4P and because of our involvement in the development of a standard document for CTCs, here the main problems related to the pre-analytical variables for LB analysis will be evidenced. The overall objective of the 48-month project SPIDIA4P is to develop selected pre-analytical CEN and ISO standard documents as well as corresponding External Quality Assurance (EQA) schemes and implementation tools needed to (1) reduce the number of sample-based diagnostic errors, (2) reduce the number of non-reproducible pre-clinical and clinical studies, and (3) improve and speed up biomarker discoveries and validations to reinforce the era of personalised medicine and innovations in patient care.

Within SPIDIA4P, dedicated standard documents focused on the pre-examination processes for specific LB components such as CTCs, exosomes and cfNAs are being produced. Since new methods are exponentially emerging and new tools will be developed to support the management of oncology patients, it is expected that the increasing knowledge on the origin and the functions of LB derived biomarkers in tumour development will lead to a constant and necessary revision of the protocols in view of future applications. Notwithstanding these considerations, posing the basis of standard procedures is particularly appropriate in this stage of development of LB applications in order to promote reliable results and enhance the diffusion of tests based on LB analysis. Here, the main pre-analytical aspects related to CTCs, cfNAs and exosomes in blood samples for LB analysis are discussed. Other body fluids, specifically urine and saliva, have been considered briefly.

The pre-analytical phase of cell-free nucleic acids analysis

The pre-analytical phase of cell-free DNA analysis

cfDNA can be found in the plasma of healthy and diseased individuals due to cell death by necrosis or apoptosis and mechanisms of active release. In cancer patients, a fraction of cfDNA is represented by DNA of tumour origin bearing the same genetic and epigenetic alterations as the tumour [14]. The major potential clinical application for cfDNA lies in the real time monitoring of therapies; in fact, genomic aberrations affecting the efficacy of targeted drugs can be identified non-invasively in cfDNA [4]. The analysis of cfDNA has been recently introduced into clinical practice for the assessment of the mutational status of the EGFR gene in patients affected by non-small cell lung cancer when tumour tissue is unavailable (https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/208065s006lbl.pdf). However, despite the growing importance of cfDNA in oncology, there is no general consensus on protocols for cfDNA analysis while there is a need to standardise both the pre-analytical and analytical procedures to obtain reliable and reproducible results in different laboratories. Accordingly, the pre-analytical phase for cfDNA analysis has been addressed by several authors (for review, see [15,16]); here, we summarise the pre-analytical factors affecting cfDNA analysis and report the most recent studies in this field.

The first issue to be considered is the choice of the matrix; although the majority of the studies on cfDNA are performed on plasma, several authors use serum as a source of cfDNA. Moreover, serum appears to have a higher content of cfDNA [15,17], but it has been demonstrated that this can be attributable to genomic DNA contamination caused by leucocyte lysis during the clotting process [15].

Secondly, blood collection tubes may have a great impact on the analytical phase. The most common collection devices are K_2/K_3EDTA-containing tubes, which require a short time interval between blood drawing and sample processing, since it has been demonstrated that leucocyte lysis occurring after draw causes an increase in DNA concentration over time. Studies on DNA concentration in K_3EDTA tubes report non-uniform results about the stability of this parameter for up to 4, 6 and 8 h from collection [16] or even over 24 h [17], making it evident that stabilising blood collection is a prerequisite for the reproducibility of the analytical data. Different collection devices with preservative reagents are now available among which cell-free DNA BCT tubes (Streck, La Vista, Nebraska, USA) have been shown to prevent genomic DNA contamination during sample storage for up to 14 days at room temperature (RT) [18]. Based on a comparison with K_2EDTA containing tubes, Streck tubes were capable of maintaining stable cfDNA levels for 7 days if stored at RT while K_2EDTA tubes under the same conditions showed a massive release of DNA [19]. Moreover, the same devices appeared to have better performance than PAXgene Blood ccfDNA Tubes (Qiagen, Hilden, Germany), which showed considerable cell lysis at 7 days storage at RT [20]. Nonetheless, PAXgene Blood ccfDNA Tubes have been demonstrated to stabilise the sample for 7 days at RT with no significant increase in the amount of DNA [21].

In addition to reducing genomic DNA contamination, Streck tubes allowed increased KRAS mutation detection by qPCR over EDTA tubes after a 72 -h incubation at RT [22]. Storage of blood for up to 5 days at RT in Streck DNA BCT tubes did not affect cfDNA extraction or the mutation background levels assessed by BEAMing and Next Generation Sequencing [23]. The Qiagen PAXgene tubes and Cell-Free DNA Collection tubes (Roche Diagnostics, Mannheim Germany) showed similar performances to Streck tubes, allowing the detection of low fractions of mutant DNA of tumour origin in a background of wild type DNA after 7 days of storage at RT [24]. A comparison of Streck, EDTA and CellSave (Veridex, Raritan, New Jersey, USA) tubes, performed by storing the blood from metastatic breast cancer patients for 2, 6 and 48 h at RT and quantifying total cfDNA and tumour cfDNA by digital PCR, revealed that tumour DNA was similar and stable for up to 6 h in all tube types, while after 48 h, a lower level of tumour cfDNA was found in the EDTA tube with concomitant increase in wild type DNA [25]. The same blood collection devices were tested in another study on subjects affected by metastatic cancer bearing a known somatic variant: 1 h after collection, the cfDNA concentration and fragment size distribution were similar in all tubes, while after 24 and 96 h, only tubes with preservative agents guaranteed unaltered cfDNA concentration and fragmentation [26].

In addition to the above-mentioned blood collection devices, new preservative tubes have been developed, such as Norgen Biotek cfDNA Preservative Tubes (Thorold, Ontario, Canada) for collection and preservation of whole blood for up to 30 days at RT, and ImproGene Cell Free DNA Tube (Improve Medical, China) stabilising cell free DNA 7-14 days under 4–30°C. However, for these new products, no evaluation/comparison study has been published to date.

The blood sample storage conditions represent another critical issue influencing cfDNA fragmentation [15]. In a recent report, Streck BCT, Qiagen PAXgene and Roche Cell-Free DNA collection tubes were tested at higher temperatures than those recommended by the manufacturers, revealing good performances for the first two devices in terms of presence of high molecular weight contaminating DNA [27]. Optimal blood processing is another prerequisite for obtaining reliable results, and it is now accepted that a double centrifugation step (the first at $1600 \times g$ and the second at $16{,}000 \times g$ for 10 min) is necessary for isolating cell-free plasma [22]. Since the centrifugation temperature may also influence plasma separation, several laboratories perform a double centrifugation protocol at 4 °C [7,28]. In addition, plasma storage times before cfDNA extraction matters among pre-analytical factors; in fact, while plasma storage for 2 weeks at −80 °C and for 4 weeks at −20 °C apparently has no effect on cfDNA extraction, as well as three freeze-thaw cycles [16], longer periods of storage at −80 °C (5–21 months) may cause a decreased cfDNA yield [29]. These results are in line with those reported elsewhere [15].

One of the major pre-analytical factors influencing cfDNA analyses is undoubtedly the choice of extraction method. There is a wide variety of methods based on different principles, such as phase isolation, silicon membrane spin columns and magnetic beads [30] and it is difficult to choose the best performing one. The major challenge is the low quantity of cfDNA circulating in the blood, demanding very high sensitivity. In early research on cfDNA, few methods specifically designed for DNA extraction from plasma were available and different protocols were adopted. This may explain the higher yield of cfDNA described for DSP virus kit (Qiagen), designed to extract viral nucleic acids from plasma, over QIAamp DNA blood Mini kit (Qiagen), which was largely used previously [16]. We described similar findings comparing the same kits, while the two protocols designed for plasma (QIAamp DSP Virus and QIAamp Circulating Nucleic Acid kit, Qiagen) showed no statistical differences in cfDNA yield [29].

One of the first assessments of the performance of different extraction methods reported a strong dependency of cfDNA yield on isolation procedure with an automated method showing better results than two different manual spin column kits [31]. We obtained similar results by comparing the on column QIAamp DSP Virus Kit with the automated protocol QIAsymphony Circulating DNA kit (Qiagen) on a limited set of samples [Pinzani et al., unpublished data]. The latter approach showed a slightly higher yield than another automated method, the Maxwell RSC ccfDNA Plasma kit (Promega, Madison, Wisconsin, USA) [Pinzani et al., unpublished data]. In another study, the QIAamp Circulating Nucleic Acid kit gave the highest DNA yield compared to DSP Virus/Pathogen Midi Kit on QIAsymphony and Analytic Jena PME free-circulating DNA Extraction Kit (Jena, Germany), with all three methods providing sufficient DNA for downstream applications [22].

Other automated sample preparation systems, InviGenius and InviGenius PLUS (Invitek Molecular, Berlin, Germany), were recently tested for their ability to recover cfDNA, which was superior to that of column-based manual isolation methods [32]. It was reported that the QIAamp Circulating Nucleic Acid kit provided the highest cfDNA yield

and low molecular weight fractions when compared with other two spin column-based methods, while among four tested magnetic bead-based methods the MagMAX Cell-free DNA Isolation kit showed the best performance [33]. The same two methods emerged as best in terms of total cfDNA concentration, cfDNA integrity and KRAS mutated cfDNA fraction in addition to another comparison among five DNA isolation kits [34]. Other authors evaluated five different DNA isolation kits prior to the digital PCR analysis of cfDNA, obtaining the highest yield with the Zymo Quick cfDNA serum & plasma kit (Zymo Research, Irvine, California, USA) and the most consistent digital PCR results emerged while using the QIAamp Circulating Nucleic Acid Kit [17].

The performance of different cfDNA isolation protocols in 56 European laboratories was evaluated in the context of the European project SPIDIA (Standardisation and improvement of generic Pre-analytical Tools and procedures for In vitro DIAgnostics) by performing an External Quality Assessment program. Participating laboratories received the same plasma sample and extracted cfDNA using their own procedures at defined plasma storage conditions. Most were able to recover cfDNA but only a few recovered non-fragmented cfDNA. Moreover, extraction methods specifically designed for cfDNA preserved the integrity profile [6].

Finally, other pre-analytical variables with potential effects on the analytical results are the storage of isolated cfDNA and the methods for cfDNA quantification, which have been thoroughly dealt with elsewhere [15,16].

Remarks

The multiplicity of reports, with sometimes contradictory results, on the pre-analytical variables for cfDNA analyses is the result of the urgent need to standardise procedures through different laboratories in view of a clinical application of LB tests. Although results are still partial and larger studies are needed to establish a consensus on all the different aspects of the workflow for cfDNA analysis, the following evidence emerges from the literature: (1) the use of preservative agents stabilising cfDNA qualitatively and quantitatively appears to be a necessary step toward the standardisation of pre-analytical variables; (2) overall automated protocols appear to have better efficiency in isolating cfDNA than manual kits; in addition, different approaches may select fragments of cfDNA with variable lengths. Thus, careful evaluation of the DNA isolation kit is needed when considering downstream analyses.

The pre-analytical phase of cell-free RNA analysis

The potential utility of cell-free RNA (cfRNA) as a cancer marker was demonstrated in different malignancies several years ago combined with the observation that RNA is much more fragile than DNA and prone to degradation by ubiquitous RNases [35]. The factors limiting the use of cfRNA in a clinical setting include degradation of cfRNA and the increasing background RNA derived from leucocytes that can hamper the detection of targets expressed at low levels [36]. Changes in cfRNA stability may occur during blood processing, shipping and storage; thus, the first step toward a standardisation of the pre-analytical variables is stabilisation of the sample at the time of the blood draw. Specific blood collection tubes containing reagents inhibiting RNases and preventing non-specific release of background RNA have been developed. These devices, Cell-Free RNA BCT tubes (Streck, USA), were shown to stabilise RNA for up to 3 days at RT, while K_3EDTA vacutainers presented a constant increase in RNA concentration in the same conditions [36]. In addition, Streck Cell-Free RNA BCT tubes were shown to minimise the increased background RNA caused by temperature fluctuations or agitation during storage and shipping [37]. The isolation of high quality, intact cfRNA is challenging, and extraction procedures can affect downstream analyses [38]. It is essential to evaluate the integrity of cfRNA after isolation in order to reach reliable gene expression results [38], which is not feasible with conventional methods, e.g. capillary electrophoresis, which lack the sensitivity to detect low amounts of circulating cfRNA. An RT-qPCR method was reported based on the ratio of the concentrations of transcript sequences corresponding to the 3′ and the 5′ end of a housekeeping gene to evaluate the integrity of plasma RNA [39].

In the last ten years, circulating miRNAs have emerged as promising, potential minimally invasive, disease biomarkers that are considered valuable in diagnosis, prognosis and treatment response monitoring. Although found to be stable in body fluids, a comprehensive overview of studies revealed considerable differences and a lack of concordance in the results. Several analytical and pre-analytical issues must be addressed before cell-free miRNAs can be validated as biomarkers [40–42] and variables influencing the accurate analysis of extracellular miRNAs have not been extensively studied [41].

Pre-analytical factors include sample related factors, such as the choice of matrix (serum or plasma), hemolysis, contamination by platelets and leucocytes and sample collection time, as well as exercise and diet in addition to procedure related factors such as sample processing, storage conditions and isolation methods [43]. Moreover, multiple methods for the extraction of circulating miRNAs are available, among which the two major categories are phenol-based techniques associated or not with silica columns and phenol-free techniques together with columns for RNA isolation [40,43].

Cell-free long non-coding RNAs (lncRNAs) have also been discovered as a new class of cancer biomarker with potential application in diagnosis and prognosis of different cancer types and predicting and monitoring treatment response. Pre-analytical aspects worth investigating include the source of lncRNAs (plasma/serum), the importance of eliminating the cellular components that could affect their quantification and the extraction methods. No systematic studies comparing different isolation methods are yet available, and mainly guanidine/phenol/chloroform-based protocols or commercial kits using columns have been used [44].

Remarks

Even if the pre-analytical phase for cfRNA analysis has so far not been extensively investigated, the same factors influencing cfDNA analysis must be considered in order to reach a standardisation of the protocols leading to reproducible inter-laboratory results.

The pre-analytical phase of CTC analysis

Circulating tumour cells, shed into the bloodstream from primary tumours and metastases, represent a promising surrogate material of the tumour, characterisation of which may provide a non-invasive approach to real-time disease monitoring in cancer patients. Hence, CTCs have been defined the ‘liquid phase of tumour progression’ [45]. In recent years, a plethora of new technologies for CTC identification, counting and characterisation has been reported. However, only a limited number were tested on cancer patients, mainly due the fact that CTC analysis is still technically challenging because of the low number of cells among a much larger proportion of normal blood cells.

The potential clinical applications of CTC analysis span from early disease detection to prognosis and identification of therapeutic targets and resistance mechanisms as well as real-time monitoring of therapies [4]. The only FDA approved clinical application for CTCs is CTC count by CellSearch (Menarini Silicon Biosystems, Bologna, Italy) in metastatic breast [46], prostate [47] and colon [48] cancer, which has been shown to have a prognostic role in these conditions. While CTCs hold promise for clinical applications focused on non-invasive disease management aimed at personalised medicine several steps must be undertaken during standardisation of the pre-analytical, analytical and post-analytical procedures to translate CTC analysis into clinical practice. Moreover, where molecular characterisation of CTCs is being pursued, analysis requires a complex multi-step process starting from blood collection and CTC enrichment, followed by CTC purification from residual contaminating leucocytes. The different approaches

available for each step render it difficult to standardise the procedures across different laboratories.

Technical advances have enabled molecular analyses at the single-cell level allowing the profiling of rare cancer cells in clinical samples, which have led to the discovery of the molecular heterogeneity of single CTCs in the blood, probably reflecting tumour heterogeneity. These findings opened a new field of research on individual CTCs (for review, see [49,50]). The analysis of nucleic acids from single CTCs adds further complexity to the experimental workflow by introducing single-cell sorting, a critical step requiring highly sophisticated technology and expertise, and whole genome/transcriptome amplification (WGA/WTA), or targeted mRNA preamplification to obtain sufficient material for downstream molecular analyses while being prone to introducing analytical biases.

While approaching the pre-analytical phase for CTC analyses, it is important to be mindful of the final goal: where the focus is CTC count and/or their morphological analysis, the pre-analytical phase starts with blood collection and includes CTC enrichment followed by sample pre-treatment prior to staining, whereas when the aim is molecular characterisation of CTCs, the pre-analytical steps comprise extraction of nucleic acids from enriched CTCs or WGA/WTA procedures when dealing with single or a few cells. Although the pre-analytical phase for CTC analysis has not been thoroughly investigated so far, some studies are beginning to emerge, and are summarised here.

Blood collection prior to CTC analysis

The first pre-analytical aspect common to all CTC analyses is sample collection. In most cases, blood is collected in K_3EDTA tubes, requiring a very short time interval between blood draw and sample processing in order to avoid cell lysis. Clots (or micro-clots) interfere with two of the major classes of CTC enrichment procedures (filtration and immunomagnetic capture); hence, specimens with evidence of clotting should be considered noncompliant.

We evidenced the fragility of CTCs in blood collected in EDTA tubes in a pilot study on the SK-MEL-28 tumour cell line spiked into the blood of a healthy donor collected in EDTA tubes and recovered by filtration after 3, 24 and 72 h. Cell morphology was preserved at least for 3 h, while after 24 h the sample showed few cells with altered morphology and after 72 h, cells emerged highly damaged [Pinzani et al., unpublished data].

A critical aspect is the low availability of blood collection devices specifically designed for CTCs; some of the tubes (e.g. Cell-Free DNA and Cell-Free RNA BCT, Streck) were initially designed to stabilise cfNAs by preventing the release of nucleic acids from cells [36,18] and were tested only later for preservation of CTCs. Additionally, even in the absence of a systematic study of the performance of different collection procedures prior to CTC enrichment, some studies comparing different blood collection devices, with or without preservatives, have been published. In one report a comparison was made of K_3EDTA (BD Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey, USA), CellSave (Veridex) and BCT (Streck) tubes prior to CTC detection and counting by CellSearch on samples obtained by spiking a breast cancer cell line into the blood of healthy donors. Tubes with preservatives (CellSave and BCTs) were able to maintain CTCs during transportation and storage at RT for up to 4 days, showing comparable recovery rates; recovery rates in K_3EDTA tubes was much lower and significantly decreased between days 1 and 4. Stability for EpCAM (epithelial cell adhesion molecule) and CK (cytokeratin) was assessed by immunofluorescence in BTCs for up to 4 days at RT, while in K_3EDTA tubes, the fluorescence signals appeared reduced due to protein degradation. In addition, CTCs from BCTs showed stable mRNA expression for two target genes, while those from K_3EDTA showed altered expression for the same targets [51].

Streck Cell-Free DNA BCT tubes were demonstrated to perform significantly better than EDTA, citrate and heparin tubes for CTC detection in samples from breast cancer patients processed with the high-definition single cell analysis assay (HD-SCA) 24 and 72 h after blood draw. The 4 tubes were tested for the possibility of generating whole-genome copy number variation profiles by NGS after single cell isolation by micromanipulation followed by whole genome amplification. Single cell genomic analysis was feasible in all tube types at 24 h and in Streck Cell-Free DNA BCT tubes at 72 h, with the most robust and reproducible results obtained for samples collected in Streck tubes at 24 h [52].

In addition, the performance of K_3EDTA tubes and Streck Cell-Free DNA BCT tubes were compared in a different experimental setting involving CTC enrichment by size using the ISET system (Rarecells Diagnostics, Paris, France) in a cohort of patients affected by non-small cell lung cancer. Streck Cell-Free DNA BCT tubes showed stable CTC counts at 24 and 48 h after the blood draw and preserved the morphology and integrity of the CTCs and leucocytes, while the K_3EDTA tubes presented CTCs and white blood cells with altered integrity after 24 -h storage at room temperature; moreover, the BCT tubes preserved the detection of predictive biomarkers, at the protein and DNA level, such as MET protein expression by immunocytochemistry and ALK rearrangement by FISH [53]. Surprisingly, based on the comparison of K_3EDTA, acid citrate dextrose-B, Cell-free DNA BCT, Cell-free RNA BCT and Cyto-Chex BCT (Streck) tubes, after CTC enrichment by immunomagnetic capture on spiked samples with a prostate cancer cell line, cell recovery was not affected by the blood tube type even after 48 h storage. On the other hand, tumour cell-specific RNA was undetectable by digital PCR in CTCs from stored blood samples containing preservatives. This was attributed to crosslinking effects suppressing RNA accessibility in tubes with stabilising reagents [54]. We have verified that Cell-free RNA BCTs allowed a reliable expression analysis by RT-qPCR of a panel of genes on CTCs enriched by CellSearch and purified by DEPArray (Menarini Silicon Biosystems) with better performance than CellSave and EDTA tubes [Pinzani et al., unpublished data].

In addition to CellSave, containing a reagent specifically designed for preservation of CTCs for up to 96 h at RT, another collection device for collection and storage of human whole blood specimens for CTC evaluation is Circulating Tumour Cell TransFix/EDTA Vacuum Blood Collection Tube (CTC-TVT, Cytomark, Buckingham,UK), which shows significant extension of the integrity of CTCs within the samples [55,56]. CTC-TVTs have been used to assess the mutational status of single CTCs from metastatic breast cancer patients after 72 h from the blood draw upon enrichment by filtration using the Screencell Cyto device (ScreenCell, Paris, France) and single cell sorting by the DEPArray system [57].

The majority of blood collection tubes with preservatives contain fixatives that kill the cells and preserve their morphology. A sugar-based cell transportation solution (SBTS, HemSol) has been formulated specifically for the storage and transport of live CTCs. The solution is capable of maintaining viable cell lines for more than 72 h at RT and permits storage of cell lines spiked into whole blood for up to 7 days. Viable CTCs from cancer patients were retrieved by CellSieve CTC microfiltration system (Creatv Microtech, Potomac, Maryland, USA) after 6 days of storage in SBTS [58].

It is evident that the standardisation of blood collection prior to CTC analysis is critical. The use of preservatives seems necessary to avoid CTC lysis, but the results on their performances are fragmented and sometimes contradictory. This could be due in part to the extreme variability of experimental approaches available for CTC enrichment, isolation and characterisation. The choice of collection tube should consider the downstream analyses and the compatibility of the reagents with each step of the complex workflow required.

Pre-analytical variables influencing CTC counting

Blood collection is only the first aspect of a complex workflow aimed at identifying and counting CTCs. The second step is CTC

enrichment, consisting of a variety of methods relying on two main approaches, recognition of marker proteins and the physical properties of the cells, each with its advantages and disadvantages, which influence the results by selecting different populations of cells (for review, see [45,59]).

Pre-analytical variables influencing molecular characterisation of CTCs

A bulk analysis of enriched CTC samples requires a nucleic acid isolation step that may impact the result. No specific studies on nucleic acid extraction from CTCs have been reported to date, but in view of a clinical application of CTC characterisation, investigations on this would be welcome. For molecular characterisation of single CTCs, two additional steps must be considered: (i) single CTC isolation, which can be achieved by manual, time consuming, low-throughput methods such as micromanipulation and laser assisted microdissection, or highly expensive, sophisticated instrumentation such as the DEPArray system and microfluidic devices; (ii) amplification of the genome/transcriptome of the single cells. A large number of WGA and WTA approaches is now available on the market but WGA is prone to errors causing false mutation calls, and WTA hardly achieves reliable amplification of less represented transcripts and may be biased to the 3′-end or the 5′-end of a transcript [60].

Remarks

Studies on the pre-analytical phase for CTC analysis are in their infancy. In order to reach a standardisation of pre-analytical conditions, it is necessary to reach a consensus on the different workflows aimed at obtaining information from CTCs (mainly CTC count or characterisation). More comprehensive studies are needed to address all the aspects of the complex pipeline.

The pre-analytical phase of exosome analysis

A new promising target of LBs involves secreted membrane vesicles, collectively called extracellular vesicles (EVs) [12] among which exosomes are usually indicated as endosomal-derived vesicles ranging in size from 30 to 120 nm and released into the extracellular microenvironment by different cell types, including tumour cells; however, this definition is still subject to change since larger exosomes (up to 250 nm) have been described and also apoptotic cells release exosome-like vesicles. Instead, the term EV is increasingly used as it encompasses all vesicle types released by cells [61]. The release of EVs from cells is an active process. They have been detected in biological fluids (blood, urine, saliva, etc.) carrying RNA, microRNAs, DNA and proteins from their originating cells [62] and mediate inter-cellular communication at both the paracrine and systemic levels. [63]. Tumour exosomes have been shown to promote tumour cell growth, immune response suppression and induction of angiogenesis [64] and play a role in metastasis [65,66]. They may be used as LB-based biomarkers [63,67] due to their tissue-specificity and ability to transport (and protect) their cargo, which is a putative molecular fingerprint of the cell of origin [63]. They have been associated with different cancer types, including pancreatic [68,69], colon [70], gastric [71], breast [72], ovarian [73] and lung [74].

The characterisation and isolation of exosomes is technically challenging: the pre-analytical variables (sample collection, storage, transport, exosome isolation, RNA/protein) may influence size, morphology, yield and exosome stability as well as analytical methods for their characterisation [75] for downstream applications such as biomarker studies [61], miRNA/mRNA profiling [76,77], proteomics and/metabolomics [78,79], functional studies (e.g. cell-cell signalling [61]) and basic biological research (e.g. role in tumorigenesis [68,69,71–74]).

The literature on exosomes and other extracellular vesicles reports a wide description of vesicle isolation and characterisation protocols as well as nomenclature, resulting in a considerable obstacle in comparing results of independent studies; in the absence of specific guidelines on the minimal information needed for EV data publication, it is often difficult to extrapolate the type of isolated vesicles (e.g. exosomes vs other vesicles) or discriminate the origin (EV or non-EV) of an isolated molecular target (e.g. microRNA). In addition, it is increasingly evident that the standardisation of procedures is strongly needed, including those for the pre-analytical workflow and data reporting, starting from sample collection to nucleic acid or protein extraction from isolated vesicles.

Sample collection, storage and processing

Collection of EV-containing fluids must be gentle to minimise cell lysis, which can lead to release of vesicles from intracellular compartments with an overall reduction of sample purity [12]. Moreover, post-phlebotomy storage of blood samples increases plasma exosome concentration over time, indicating a release of exosomes by blood cells during storage [80,81]. Due to the processes mentioned above, sample stabilisation is the first step towards standardisation of the pre-analytical workflow, despite there being currently no specific EV stabiliser commercially available. In order to minimise vesicle release from blood cells after phlebotomy, particularly from platelets, most of the reported procedures are based on the quantification and characterisation of circulating microvesicles in plasma rather than whole blood, using sodium citrate [82,83], acidic-citrate-dextrose [13] and EDTA [84] as anticoagulant, or serum [76,84,85]. However, storage time before centrifugation, transport (particularly sample shaking and physical stress), choice of the centrifugation protocol and storage temperature are reported to impact on downstream analysis [84,86–88].

Exosome isolation

Exosome isolation from biofluids is a challenge. EVs are a complex and heterogeneous class of vesicles; an isolated EV preparation generally contains a mixture (including exosomes, microvesicles and apoptotic bodies), but a marker classification to distinguish EV subsets has not been established [13,63,89]. No consensus has been achieved on a ‘gold-standard’ method to isolate and/or purify exosomes and to provide preparations with a substantial yield of reliable quality; the choice may depend both on the specific purpose and the downstream applications used in addition to sample volume and desired degree of purity [12,63].

Differential ultracentrifugation methods

These techniques (coupled or not with density gradients) are the most commonly used and standardised procedures. The methods (by which large sample volumes can be processed) allow both large EVs (mostly microvesicles) and small EV (mostly exosomes) enriched preparations to be obtained by using different centrifugal forces (10,000 x g and 100,000 x g, respectively) [90,91]. A series of guidelines has been proposed for EV isolation and characterisation from cell culture supernatants and biological fluids [92], after which the described protocol was further optimised [90,93,94]. Furthermore, in order to increase the purity of the isolated EVs, ultracentrifugation can be performed on sucrose or idioxanol density gradients; by this procedure the elimination of non-EV material or fragments due to damage (vesicles breakage, fusion or aggregation during ultracentrifugation) can be achieved [63,95]. Although a large number of exosomes can be obtained, ultracentrifugation presents several technical limitations such as appropriate equipment requirements, time-consuming workflow and lack of automation [95].

Filtration systems: size exclusion chromatography (SEC) and ultrafiltration

Exosome isolation is based on the size (or molecular weight) differences among distinct types of EVs. In particular, SEC has a low impact on EV integrity, allows preservation of EV function [96,97] and has been applied successfully for the analysis of plasma EVs [98].

Ultrafiltration is fast and does not require expensive equipment. Both ultrafiltration and SEC can remove contaminants (e.g. proteins) and can be applied downstream of other enrichment methods [63]; but relatively small sample volumes can be processed.

Polymer-based isolation systems for exosome precipitation

The use of a polymer that alters the exosome solubility (or dispersibility) induces exosome precipitation; subsequently, exosomes are isolated using low-speed centrifugation or filtration [99]. Different exosome precipitation kits are available, among which Exoquick (System Biosciences, Palo Alto, California, USA [100]) and Total Exosome Isolation kits (Life technologies, Carlsbad, California, USA [101]) are probably the most used. Precipitation is easy to use, does not require specialised equipment and is scalable to large sample sizes. However, the possibility of co-precipitation of non-exosomal contaminants (e.g. proteins) affecting the purity of the preparation exists.

Immunoaffinity methods

Affinity methods specifically separate EVs through interactions between membrane antigens and immobilised antibodies or membrane receptors and their ligands. There are several immunoprecipitation kits targeting different proteins (e.g. CD81 or CD63, [102,103]) as well as ELISA-based methods [104]. These rather expensive techniques isolate specific exosome populations and subtypes (even with a low yield recovery, perhaps due to epitope blocking or masking [105]) with high purity [106].

Microfluidics-based methods

These techniques use microfluidic devices relying on both physical and biochemical properties of exosomes such as immunoaffinity size and density. Exosearch [107] and Immunochip [108] have been used for specific quantification of circulating exosomes, allowing a quick and efficient isolation, with a significant reduction in sample volume and reagent consumption, even if the procedure is far from fully standardised [106].

Intact exosome analysis

Visual identification of exosomes can be carried out to confirm their presence and purity of the preparation and is usually performed by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Flow cytometry in combination with specific antibodies for the analysis of exosome subsets can also be used as quality control. These techniques require dedicated, expensive instrumentation and are mainly applied to exosome characterisation studies.

Exosome cargo analysis

Isolation of exosomal RNA/proteins: EV RNAs, combined with non-EV RNAs, are a very interesting target for LB analysis. Non-EV RNAs are unstable in blood due to the exposure to RNases and RNA profiles can change significantly after blood collection [109–112]. In contrast, EV-RNA, as part of the vesicle cargo, is protected from enzyme activity. However, not all stable RNA in biofluids is contained within vesicles; in body fluids (especially serum and plasma) extracellular RNA (including microRNA) can be transported on non-EV carriers, including protein complexes (AGO2) [113] and lipoproteins (HDL and LDL [114]). The RNA content of EVs has been analysed using RNA-seq, hybridisation arrays and other methods, revealing the full spectrum of previously known transcripts including miRNAs and other species of small ncRNAs such as Piwi-interacting RNAs (piRNAs) as well as mRNAs, tRNAs, lncRNAs and rRNAs [115,116]. Evidence of vesicle-specific modification, enrichment and isoforms of the enclosed RNA [115,117,118] has also been reported.

For downstream analysis of exosomal content, several alternative RNA extraction methods have been described, including phenol-based techniques (TRIzol) and combined phenol and spin column-based techniques (miRNeasy Mini Kit, Qiagen, and mirVana miRNA Isolation Kit Thermo Fisher Scientific, Waltham, Massachusetts, USA) [119]. Other kits — SeraMir Exosomes RNA Amplification kit (SeraMir), Total Exosomes RNA and Protein Isolation kit (Thermo Fisher Scientific) — have been designed specifically for isolation of RNA and protein from a pre-enriched exosome preparation. Kits to obtain EV-RNAs directly from serum or plasma, e.g. exoRNeasy Serum/Plasma kit (Qiagen) [120], are also available. They use a spin column format and dedicated buffers to purify exosomes from prefiltered plasma; they include an exosome purification stage, in which prefiltered plasma is mixed with a dedicated buffer and bound to a membrane affinity spin column, and an RNA extraction step. Total RNA, including miRNAs, bound to the spin column is then eluted.

Isolation of exosomal miRNAs: among the various molecules contained in exosomes, microRNAs are of special interest because they have been found to regulate multiple genes and gene expression in cancer [121]. It has been demonstrated that the profile and concentration of miRNAs are different among the intracellular, cell-free and exosomal components of blood [76,77]. The persisting technological challenges in profiling exosomal miRNAs are mostly due to the lack of standardisation of the pre-examination process, including miRNA isolation.

It has been shown that the use of different purification procedures can slightly affect exosomal contents, including miRNAs [122,123]. The choice of the extraction method can depend on the exosome isolation method used, since a dedicated kit for miRNA isolation could be specified by the isolation provider as part of a validated workflow. Kits for miRNA or total RNA (including miRNAs) extraction from enriched preparations includeTotal Exosome RNA and Protein Isolation Kit-Thermo Fisher Scientific or Exosomal RNA Isolation kit-Norgen) as well as miRNA extraction kits from unenriched samples (e.g. plasma or serum).

Remarks

The pre-analytical phase of EVs (analogous to that of other components of the LB) will demand a careful analysis of EV enrichment and isolation steps in accordance with the requirements of the analytical phase that can be oriented to reach different endpoints and information. Thus, a standardisation process with the aim of harmonising procedures focused on different applications is needed. Clarification of the nature and origin of the EVs can help in the design of the most suitable procedure, in view of the use of nucleic acid and protein cargo as the source of important biomarkers to be applied in oncology as diagnostic/prognostic markers, supporting development of personalised treatments.

The pre-analytical phase of liquid biopsy in body fluids other than blood

The term ‘liquid biopsy’ mostly refers to blood as a source of cfNAs, CTCs and exosomes. However, recent studies have reported the use of other body fluid-based LBs, e.g. saliva and urine [124,125], for the detection of tumour components or cancer related molecular markers such as circulating tumour DNA (ctDNA) [126–128], exosomes [129,130] and miRNAs [131] and their application in cancer diagnosis, screening and monitoring [131,132]. Biofluid-based LBs potentially offer advantages over blood due to non-invasive features and proximity to the tumour [125]; the collection of urine and saliva (the most frequently used biofluids) is completely non-invasive, relatively safe, economic and, with proper instruction, can be performed at home without educated professionals or dedicated facilities. Tumour specific gene alterations usually detected in plasma ctDNA have also been found in urinary ctDNA of cancer patients [133,134]; according to ref [126], saliva samples are preferentially enriched for tumour DNA from the oral

cavity in comparison to plasma samples in patients with head and neck squamous cell carcinomas (HNSCC). These findings highlight the potential clinical relevance of analysis of specific body fluids depending on the anatomical location of the tumour in order to improve sensitivity. Non-blood body fluids can also be used as source of tumour cells contained in a sediment after specimen centrifugation. However, they are not considered in this review, which is focused on the pre-analytical aspects related to the analysis of circulating biomarkers.

As for blood samples, analysis of cellular and acellular components of urine and saliva can be compromised by sample handling, shipping and processing. Moreover, stabilisation of cellular and circulating components, including red and white blood cells, cfNAs and microvesicles, at the time of collection is needed to preserve the integrity of sample components for downstream applications. Sample collection performed without a specific stabiliser can lead to lysis of nucleated blood cells and subsequent release of contaminating genomic DNA; the degradation of cfDNA due to nuclease activity can also occur. In addition, bacteria present in the specimen can continue to grow after collection, diluting human NA content and/or accelerating its degradation with an impact on the sensitivity and reliability of molecular examination.

Some collection devices with dedicated preservatives for ctDNA are available for saliva and urine (e.g. RNAPro•SAL Split Sample Kit for LB, Oasis Diagnostics, Vancouver, Washington, USA, Cell free DNA Urine Preserve, Streck) as well as other preservatives for different biofluids for downstream molecular examination, RNA/microRNA/DNA/Proteins (e.g. Norgen's Urine Preservative; Stool Nucleic Acid Collection and Preservation Tube Norgen; GeneFix RNA/DNA Saliva Collection, Boca Scientfic, Westwood, Massachusetts,USA; DNA saliva collection, DNA Genotek, Ottawa, Ontario, Canada). Since salivary and urinary cfDNA and microvesicles are present at low concentration in the specimens, their presence being influenced by the localisation of the primary tumour and metastatic lesions [126,131], the choice of isolation and enrichment procedures, which have to be compatible with the preservative reagents or any pre-treatment of the samples (e.g. saliva filtration), is crucial for successful downstream examinations [135]. After collection, samples are usually enriched by centrifugation in order to collect the supernatant and remove the cell pellet. Kits for urine and saliva cfDNA extraction as well as exosome isolation are available [135,136].

cfDNA and exosome cargo quantification (usually miRNAs) can be technically challenging due to the low amounts of the target molecules; the quantification procedures adopted should be suitably sensitive and specific (e.g. ddPCR, fluorimetric assays). The pre-analytical considerations for methods of cfDNA quantification and storage of isolated cfDNA (both with a potential impact on analytical results) have already been reported above and discussed in [15,16]. A recent study has demonstrated tumour DNA in sputum, stool, cerebrospinal fluid (CSF) and pleural fluids along with their potential application to diagnosis and monitoring of cancer progression [125].

Remarks

Non-blood cell-free biomarkers have great potential in oncology for diagnosis, screening and monitoring of cancer progression because of non-invasive features and ease of access. The direct contact of cancer cells and body fluids may help the detection of circulating biomarkers compared with plasma by increasing the sensitivity of the detection. Many issues have to be tackled, including standardisation of specimen collection procedures (e.g. first morning urine, first voided), definition of instructions for home collection, specimen stabilisation, choice of suitable and compatible enrichment/isolation method, definition of quality assessment for the isolated biomarkers and EQA implementation. The improvement and standardisation of a complete diagnostic workflow is the key for broader use of LB in patient management.

Conclusions

An increasing number of applications in oncology involve the use of LB samples, but important technical issues have still to be addressed both in the analytical and pre-analytical settings [137]. Since the latter aspect has not yet been extensively addressed, this review provides a definition of the pre-analytical workflow of LB samples for different molecules and/or cellular and extracellular components (cfNAs, exosomes, CTCs) recently investigated as tumour-specific biomarkers in blood and other fluids with a potential clinical application in precision medicine.

The entire pre-analytical workflow includes specimen collection, stabilisation, transport, enrichment, storage and processing, as well as the isolation and the quality assessment of the analyte. Although specimen quality is critical for success of subsequent LB analyses, the specific parameters to assess it before processing have not been defined, apart from generic factors such as hemolysis (which can impact all LB components to different extents) and more specific ones such as blood clotting for CTC samples and residual platelets or cell debris for plasma EVs and cfNA analyses respectively [16,138]. The main challenge in LB analysis is to preserve the target cells/molecules/vesicles under investigation while maintaining the integrity of the blood cells to prevent them from releasing their contents (e.g. genomic DNA, vesicles), which can contaminate the specimen and interfere with downstream analyses.

Despite several research studies exploring clinical applications of LB, the majority of LB assays still lack evidence of clinical validity and, in particular, clinical utility [137] mainly due to the absence of standardisation of the pre-analytical phase. The major challenges are low amount, fragmentation and intrinsic instability of cfNAs, difficult isolation of tumour specific EVs, and low numbers, heterogeneity and the absence of standardised, high throughput procedures for CTCs [137]. Regarding cfNA and EVs, the identification of appropriate reference genes and/or an agreement on normalisation procedures to be adopted to guarantee a unique expression method for the results is still missing, particularly for body fluids other than blood.

Clinical validation studies, regulatory guidelines, dedicated EQAs and reference material are still lacking in this field. A specific CEN (European Committee for standardisation) Technical Specification for handling and processing of blood specimens for cfDNA analysis has been published (CEN/TS 16835:2015) within the FP7 SPIDIA project. The SPIDIA4P international consortium (www.spidia.eu/) is currently involved in the implementation of CEN and ISO standards dedicated to specific LB samples and analytes, and other international networks, such as CANCER ID (https://www.cancer-id.eu/), are also focusing on the development and validation of LB assays.

In the near future, multiple parameter approaches integrating emerging LB analytes with existing circulating biomarkers from the same specimen may improve diagnostic sensitivity and specificity of analytical tests in cancer diagnosis and monitoring. In order to reach this goal, validation of the entire workflow, including the pre-analytical phase, by the adoption of regulatory guidance to define assay performance is mandatory and will contribute to establishing clinical validity and utility of the liquid biopsy.

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