

Clinical and Investigational Applications of Liquid Biopsy in Non-Small Cell Lung Cancer

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ABSTRACT

Quite frequently it is difficult to obtain tissue biopsies in non-small cell lung cancer (NSCLC) patients; in some cases they are not even accessible. However, international guidelines recommend repeated biopsies prior to making a therapeutic decision. The “liquid biopsy” tries to integrate different approaches to address the use of peripheral blood as the source of molecular information through different types of materials and techniques, resolving these limitations and being a subrogate source of tumor information, offering the opportunity to increase the knowledge of the pathophysiology of lung cancer as a dynamic disease and into the process of metastatic dissemination. Circulating nucleic acids as ctDNA or exosomal RNA as well as CTCs or tumor-educated platelets offer the potential for early diagnosis, metastatic progression, stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms, and understanding metastasis development in cancer patients.

Key words: Liquid biopsy; Non-small cell lung cancer; Circulating tumor cells; ctDNA; Exosomes

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INTRODUCTION

In Oncology, the evaluation of response to treatment as well as accurate predictions of survival are key factors for the effective control of the disease as well as to design and develop future treatments. However, up to now, the methods we use to represent this response are relatively poor and, in the majority of cases, we can only wait for a period of time from the administration of the treatment to correlation in an image which is not always accurate in determining the extent of disease.

Additionally, quite frequently it is difficult to obtain tissue biopsies in non-small cell lung cancer (NSCLC) patients; in some cases they are not even accessible. However, international guidelines recommend repeated biopsies prior to making a therapeutic decision and it is imperative that therapeutic biomarkers are detected as early as possible so as to allow a potentially successful change in the course of treatment. The “liquid biopsy” tries to integrate different approaches to address the use of peripheral blood as the source of molecular information through different types of materials and techniques, resolving these limitations and being a subrogate source of tumor information, offering the opportunity to increase the knowledge of the pathophysiology of lung cancer as a dynamic disease and into the process of metastatic dissemination.

The study of molecular alterations in the liquid biopsy not only allows us to identify, noninvasively, candidate patients to receive the correct and more precise therapies, but it allows for closer monitoring of treatment, as plasma samples can much more easily be obtained in each patient's visit than for example an imaging test such as PET or CT⁽¹⁻⁴⁾. On the other hand, several studies have shown that the ability of radiological studies and its criteria to predict the survival of cancer

patients in the long term is limited^[5,6].

However, the achievement of new biopsies at the time of relapse is limited due to the accessibility of the metastasis, the need of invasive procedures or the quality of the biopsy specimen. Moreover, in some cases, the patient is unwilling to undergo an invasive procedure. In this sense, liquid biopsy might overcome such limitations as it is non-invasive blood test that detects circulating tumor cells (CTCs) or fragments of circulating tumor DNA (ctDNA) that are released into the blood stream from the primary tumor and from metastatic sites, being an attractive alternative for mutation search and testing.

Circulating nucleic acids as ctDNA or exosomal RNA as well as CTCs or tumor-educated platelets offer the potential for early diagnosis, metastatic progression, stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms, and understanding metastasis development in cancer patients^[7-9].

Despite these components have been mainly obtained from blood, they have also been isolated from almost all body fluids (blood, serum, plasma, saliva, urine, etc)^[10].

CIRCULATING TUMOR CELLS (CTCS)

Tumor cells are shed from the primary and the metastatic sites into the bloodstream. They have been demonstrated in the blood of patients with various solid tumors and they have been associated with poor outcome in metastatic NSCLC patients among other tumors^[10,11]. CTCs have long been considered to reflect tumor aggressiveness. In this regard, the decrease in the number of CTCs during different treatments (surgery, chemotherapy, radiotherapy, etc) has been associated with radiographic tumor response while increase in the number of cells has been correlated with tumor progression^[12].

CTCs detection and characterization represent a major challenge as they may comprise both the phenotypic and genetic information of the primary tumors. As CTCs are infrequent, appearing at an estimated level of one against the background of millions (10^6 – 10^7) of surrounding normal peripheral mononuclear blood cells in patients with metastatic carcinomas, their detection and characterization require highly sensitive and specific methods. These methods should combine isolation and detection strategies^[13]. So far, various techniques have been applied for CTC isolation and enumeration.

There is only one platform, named CellSearchTM, approved by the U.S. FDA (Food and Drug Administration) in this regard. This technology utilizes EpCAM-coated magnetic beads to isolate CTCs in several types of carcinomas in spite of limited detection efficiency. Krebs *et al* published a detection rate of 32% among patients with metastatic NSCLC disease, having ≥ 2 CTCs before chemotherapy treatment^[14].

Microfluidic platforms, as the 'CTC-chip', have been shown to be capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with EpCAM-coated micropores under precisely controlled laminar flow conditions, and without requisite pre-labelling or processing of sample. These chips had a sensitivity of almost 100% patient samples analyzed, and an average purity of 52%^[15].

Most importantly, the sensitivity of these EpCAM-dependent devices may be limited, due to the epithelial-mesenchymal plasticity, as CTCs lose their epithelial markers. Therefore, several EpCAM-independent isolation techniques have been tested based

on their physical properties, with size-based properties (e.g., ISET, isolation by size of epithelial tumor cells) or density centrifugation steps (e.g., FICOLL)^[16,17]. In this sense, a recent paper reported that CTCs could be detected in 80% of peripheral blood samples collected from 40 chemo-naïve, stages IIIA to IV NSCLC patients using ISET compared with 23% using CellSearchTM. A subpopulation of CTCs isolated by ISET did not express epithelial markers and circulating tumor microemboli (clusters of ≥ 3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearchTM. Authors concluded that both techniques should be performed together to allow more complete exploration of CTCs^[18].

Postoperatively, the eradication or decrease of CTCs following treatment is associated with improved clinical outcomes [19-20]. A recent work has analyzed also the kinetics of CTC in a prospective study where blood samples of 56 patients for CTC analysis were obtained before and one month after surgery. Their results showed that presence of CTCs after surgery was significantly associated with early recurrence ($p = 0.018$) and a shorter disease-free survival (DFS) ($p = 0.008$)^[21].

The influence of radiotherapy (RT) on the kinetic of CTC has also been reported. Dorsey *et al*^[22] investigated the change of CTC number in patients with localized NSCLC undergoing radiation treatment. Using a telomerase-based detection assay, 65% of the 30 patients were positive for CTCs prior to treatment. CTC numbers significantly reduced after radiation (9.1 vs 0.6 CTCs/ml; $p < 0.001$). This study suggested that analyzing CTC appear to reflect response to RT in patients with localized NSCLC.

For metastatic stages, a number of studies have reviewed its prognostic significance, concluding that baseline CTC count is an independent negative prognostic factor for NSCLC. Krebs *et al*^[14] showed median overall survival of 8.1 months and 4.3 months ($p < 0.001$) for patients with stage III or IV NSCLC using CTC cut-offs of <5 vs ≥ 5 respectively and with corresponding progression free survival (PFS) of 6.8 and 2.4 months ($p < 0.001$), using the CellSearch system. In multivariate analysis, CTC number was the strongest predictor of overall survival (OS) (HR 7.92; 95% CI 2.85–22; $p < 0.001$) exceeding the traditional risk factors of stage and performance status. Another study of 46 patients, with newly diagnosed or recurrent NSCLC, where CTC were measured at baseline in all patients, and in 23 patients CTCs were also measured before every chemotherapy cycle. A baseline CTC count of more than eight prior to chemotherapy was a strong predictor of reduced PFS ($p = 0.018$) and OS ($p = 0.026$). However, no correlation was observed between CTCs count and tumor size after two chemotherapy cycles^[23].

As a potentially useful tool for clinical practice, several studies have provided evidence between decreases in CTCs counts and radiographic response by either FDG-PET/CT or RECIST to predict DFS and OS. In a phase II clinical trial of erlotinib and pertuzumab, higher baseline CTCs counts were statistically associated with response to treatment by RECIST, and decreased CTC counts upon treatment were significantly associated with FDG-PET and RECIST response and longer PFS^[24]. Nevertheless, a study subsequently published failed to demonstrate prior results, probably because of the analysis of cohort of patients with different stages and histologies, both of which can confound the interpretation of FDG uptake and CTC analysis^[25].

These papers concluded that CTCs might stand as prognostic markers in cancer patients and, in the future, may help in therapy decision making, especially to select patients at a higher risk of relapse who might benefit from adjuvant therapies.

The development of personalized medicine with several targeted therapies has been associated with an increased interest in CTC isolation and characterization. EGFR mutations analysis has been examined in CTC from advanced NSCLC patients, identifying both sensitizing mutations or the T790M resistant mutation. Marchetti *et al.*^[26] identified for the first time, using the CellSearch System coupled with next-generation sequencing (NGS), EGFR mutations in CTCs in 84% of the patients corresponding to those present in matching tumor tissue. The majority of patients carrying sensitive EGFR mutations whose disease responds to drugs eventually develop resistance to these EGFR-TKIs. The T790M gatekeeper is a mutation of acquired resistance to TKIs. The development of third-generation EGFR inhibitors capable of overcoming T790M-associated resistance has led to a need for noninvasive methods of T790 detection to guide the selection of therapy^[27]. A recent work comparing the T790M genotyping from tumor biopsies with analysis of simultaneously collected CTC and ctDNA, the resistance-associated mutation was detected in 47% to 50% of patients using each of the genotyping assay, with concordance among them ranging from 57% to 74%^[28]. However, in those patients in whom the concurrent biopsy was negative or indeterminate, the two CTC and ct-DNA-based assays together enabled genotyping in 35% of these patients.

Detection and monitoring of NSCLC-specific rearrangements have also been reported. The diagnostic test for ALK rearrangement in NSCLC for crizotinib treatment has been evaluated by using a filtration enrichment technique and filter-adapted fluorescent *in situ* hybridization (FA-FISH). All ALK-positive patients had four or more ALK-rearranged CTCs per 1 mL of blood (median, nine CTCs per 1 mL; range, four to 34 CTCs per 1 mL). No or only one ALK-rearranged CTC (median, 1 per 1 mL; range, 0 to 1 per 1 mL) was detected in ALK-negative patients. This assay enabled both diagnostic testing and monitoring of crizotinib treatment^[29].

Other crizotinib target is ROS1 rearrangement. These rearrangements have been evaluated in CTCs using ISET and FA-FISH in a small group of 4 patients whose tumor had these genetic aberrations. In this study, ROS1-gene alterations were detected in every patient analyzed. Tumor heterogeneity, assessed by ROS1 copy number, was significantly higher in baseline CTCs compared with tumor biopsies in those patients experiencing progression disease or stable disease^[30].

Less common mutations have also been analyzed. BRAF is a rare oncogenic driver that is mutated in 2% of NSCLC and responds to BRAF inhibitors. A recent paper has reported on the feasibility of digital PCR to detect circulating BRAF-mutated DNA on both ctDNA and DNA extracted from CTCs in patients during targeted treatment for BRAF-V600E-mutated lung adenocarcinoma. These case series concluded that variations in BRAF-mutated ctDNA were correlated with a response according to RECIST criteria, with ctDNA being much more sensitive than CTCs^[31].

In addition to the gain of prognostic information, CTCs as a biomarker are still not used in the routine clinical practice. Further validation in large multicenter studies to evaluate reproducibility, specificity and sensibility should be prior performed.

CIRCULATING FREE TUMOR DNA

Cell-free tumor DNA (cftDNA) exists in plasma or serum and is single- or double-stranded DNA. Most cell free DNA (cfDNA) is reportedly fragmented but is a potential tool to enable non-invasive diagnostic tests for personalized medicine in providing similar

molecular information as the one derived from invasive tumor biopsies. Early studies showed that many cancer-associated molecular characteristics, such as single-nucleotide mutations, methylation changes and cancer-derived viral sequences, can be found in the cfDNA. These findings were significant for the development of ctDNA detection technology. Nevertheless, ctDNA-based assays are confronted with several challenges. Body cells release cfDNA into the bloodstream, but the majority of cfDNA is often not of cancerous origin, making it sometimes difficult to detect^[12]. Also, prior knowledge about particular mutations is usually required, which may be hard to obtain. However, recent technological advances have overcome these restrictions, making it possible to identify both genetic and epigenetic alterations^[32].

The fraction of cfDNA that is tumor derived in patients with cancer has a variable contribution ranging from < 0.1% to > 10% of the DNA, whose variability is thought to be affected by tumor burden, stage, cellular turnover, accessibility to circulation and factors affecting blood volume. The ctDNA thus released will load the same genetic (somatic) alterations as tumor cell and this genetic load may be detected and quantified. These platforms are real-time quantitative PCR (qPCR); digital PCR; Beads, Emulsion, Amplification and Magnetics (BEAMing); and Next-Generation Sequencing (NGS). The sensitivity ranges of these techniques range from 15% to 0.01%, but one of the weak points is the lack of standardization, in order to understand their clinical applicability^[33].

For years, real time qPCR has been used for the identification of genetic alterations and nucleic acids quantification. While qPCR is a standardized, relatively inexpensive technique, its precision for quantifying rare allele or genetic alterations present at very low levels is significantly lower compared to other techniques^[34]. However, most of the published studies adopted this technology for the analysis of ctDNA in lung cancer.

On the detection of the EGFR mutations in cfDNA using qPCR, Kimura *et al.* showed for the first time that plasma-derived EGFR genotype is predictive of subsequent clinical response to an EGFR-TKI. In the cases analyzed by both sequencing of the primary tumor and Scorpion ARMS assay of plasma, 72.7% of cases were concordant^[35]. As well, Mok *et al.* reported a concordance between tissue and blood tests of 88%, with blood test sensitivity of 75% and a specificity of 96% using the cobas blood test, based on qPCR^[36]. EGFR oncogenic mutations have also been analyzed from plasma of patients included into several different trials^[24,37]. The sensitivity of ctDNA in identifying EGFR mutations compared with the tissue ranged from 43 to 100%, being capable to identify EGFR mutations in patients with insufficient tissue.

The use of digital PCR has the potential for unparalleled precision enabling accurate measurements for low frequency genetic alterations quantification. dPCR allows for the detection of mutated cfDNA in a high background of wild type cfDNA and allows for small fold change differences to be detected. Several studies have assessed the feasibility of dPCR for biomarker testing and cancer monitoring and have evidenced that dPCR is indeed an adequate technologies for such purpose^[38-40].

BEAMing is based on a combination of emulsion dPCR with magnetic beads and flow cytometry for the highly sensitive detection and quantification of mutant tumor DNA molecules^[41]. BEAMing is a sensitive but complex method to detect known genetic mutations, specially when they are at a very low copy number^[42]. This technology has been compared to mutation analysis of tumor tissue in a recent publication, testing 21 mutations in BRAF, EGFR, KRAS and PIK3CA with different cancers (13.8% of NSCLC). Results

were concordant for archival tissue and plasma cfDNA in 915 cases for BRAF mutations, in 99% cases for EGFR mutations, in 83% cases for KRAS mutations and in 91% cases for PIK3CA mutations, demonstrating that BEAMing is feasible and highly concordant with tumor tissue analysis^[43].

Taniguchi *et al*^[3] used this technology for NSCLC patients to query for the evaluation of plasma testing for EGFR mutations in 44 patients with EGFR mutant NSCLC (23 with progressive disease after TKI and 21 TKI-naïve). Sensitizing mutations were detected in 72.7% of these patients, while T790M was detected in 43.5% of those with a progressive disease.

To assess the ability of different technology platforms to detect EGFR mutations, including T790M, from ctDNA, a comparison of multiple platforms was undertaken^[44], including two non-digital platforms (cobas® EGFR Mutation Test and theascreen™ EGFR amplification refractory mutation system assay) and two digital platforms (Droplet Digital™ PCR and BEAMing digital PCR). For the T790M mutation, the digital platforms outperformed the non-digital platforms. Recent publication comparing also BEAMing and cobas test, showed a higher positive percent agreement between BEAMing plasma and tumor results than cobas EGFR plasma and tumor results (82% for activating mutations and 73% for T790M using BEAMing vs 73% and 64%, respectively, using cobas plasma test)^[45].

The emerge of new drugs that target other genomic alterations rather than EGFR mutations have significantly increase the complexity of biomarker testing. In the context of an expanding number of driver mutations to test, NGS presents itself as a suitable technology since it allows simultaneous detection of multiple alterations in a very efficient manner. However, the use of this sequencing technology might be limited by a modest sensitivity. In this way, Uchida *et al.* reported a diagnostic sensitivity and specificity for exon 19 deletions of 50.9% and 98%, respectively; and for the L858R mutation of 51.9% and 94.1%, using deep sequencing as a detection system for EGFR mutation in ctDNA. As they analyzed patients of every stage, the overall sensitivity was 54.4% for all cases; stages IA-IIIa, 22.2%; and stages IIIB-IV, 72.7%, highlighting that NGS might be restricted to advance disease^[46]. Similarly, Couraud *et al* used the IonTorrent Personal Genome Machine for the deep sequencing of the most relevant hotspot somatic mutations (EGFR, BRAF, KRAS, HER2, and PIK3CA) in tumor DNA (tDNA) and plasma cfDNA of never-smoker NSCLC patients. In ctDNA, 50 mutations (36 EGFR, 5 HER2, 4 KRAS, 3 BRAF, and 2 PIK3CA) were identified. Sensitivity of the test was 58% and the estimated specificity was 87%^[47]. Because sensitivity appears to be compromised in early stages several strategies to solve this issue are under development. In this way, Newman *et al.* have developed an ultrasensitive method for quantifying ctDNA capable to detect ctDNA in every patient with stage II-IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to 0.02%. Researchers communicated that ctDNA levels correlated with tumor volume and distinguished between residual disease and treatment-related imaging changes, and measurement of ctDNA levels allowed for earlier response assessment than radiographic approaches, facilitating personalized cancer therapy^[48]. However, low quantities of cfDNA in the blood and sequencing artifacts could limit analytical sensitivity of this methodology.

To date, there have been communicated a high number of reports presenting the different clinical applications of ctDNA as liquid biopsy, specially, monitoring tumor burden, detecting resistance mechanisms, predicting response to treatment or detecting mutations

in the absence of tissue^[28]. Two meta-analysis have explored the diagnostic value of cfDNA for the detection of EGFR mutation status^[49-50], both communicating a similar effectiveness (sensitivity of 67.4% and specificity of 93.5% vs sensitivity of 62% and specificity of 95.9%).

EXOSOMES

Exosomes have emerged as a novel mode of intercellular communication. Recent studies suggest that the secretion of functional mRNA and miRNA from one cell to another is, in part, mediated through exosome-mediated mechanisms^[51].

Exosomes are involved in tumor initiation, growth, progression, metastasis, and drug resistance by transferring oncogenic proteins and nucleic acids. Therefore, exosomes and their load could be diagnostic, prognostic and predictive of responses biomarkers^[52].

These vesicles of endocytic origin transfer information to the target cells, including proteins, DNA, mRNA, as well as non-coding RNAs, through at least three mechanisms: receptor-ligand interaction, fusion with the plasma membrane, or endocytosis by phagocytosis^[53]. Thakur *et al* showed evidence that tumor-derived exosomes carry double-stranded DNA, showing that exosomal DNA represents the entire genome and could reflect the mutational status of parental tumor cells^[54].

Diverse technologies have been used for their isolation, as the magnetic activated cell sorting or ultracentrifugation, combined with sucrose gradient. Likewise, several methods, (e.g., transmission electron microscopy, Western blot, and FACS) have been used to characterize the isolated exosomes based on their biochemical (morphology, size or exosomal markers). However, there is a lack of the accurate method to determine the concentration of exosomes. The researchers have to rely on inaccurate measurements of protein concentration or nanoparticle tracking analysis. Quantitative RT-PCR, nucleic acid sequencing, Western blot, or ELISA have been used for exosome RNA and protein identification^[55].

Circulating levels of tumor exosomes, exosomal small RNA and specific exosomal miRNA have been tested as diagnostic and prognostic markers in patients with adenocarcinoma^[56].

A recent work^[57] suggested that protein concentration of circulating exosomes and miRNA levels, using a 12 specific miRNA signature previously described to be elevated in lung cancer patients^[58], are significantly higher in lung adenocarcinoma than in a control group.

The work also showed a similarity between the circulating exosomal miRNA and the tumor-derived miRNA patterns, suggesting that circulating exosomal miRNA might be useful as a screening test for lung adenocarcinoma. Similarly, another recent work reported a model based on microRNAs derived from circulating exosomes capable to discriminate between lung adenocarcinoma and granuloma^[59], but further validation is needed to confirm the predictive power of this model.

Exosomal RNA appears to be a promising source for the identification of large rearrangements in liquid biopsies, such as the EML4-ALK translocation or ROS1 translocation, both being targets for therapy with crizotinib when found in cancer tissue.

Brinkmann *et al* have recently communicated the technical feasibility to detect EML4-ALK fusion transcripts in plasma samples from patients known to be positive by tissue FISH testing using exosomal RNA. The diagnostic test they explored, named ExoDx Lung (ALK) had a sensitivity of 88% and a specificity of 100%^[60].

As exosomes are accessible in early all body fluids, urinary exosomes have been also screened as novel diagnostic indicators for

NSCLC patients. Li *et al.* have detected a higher level of expression of leucine-rich α -2-glycoprotein (LRG1) in urinary exosomes by Western blot, further validated also in lung tissue by IHC, between NSCLC patients and healthy controls. These results suggested that LRG1 could be a candidate biomarker for non-invasive diagnosis of NSCLC in urine^[61].

However, despite all the evidence, the exact mechanisms mediating the clear roles of exosomes in cancer have not yet been fully elucidated.

DISCUSSION

We believe that the monitoring of cancer through different liquid biopsy methodologies, together with correlation of more accurate imaging techniques (PET/CT and Radiology with functional CT and MRI), and adapting these to the new technologies already present in the routine clinical practice, will allow us to more precisely determine the response to treatment. Several studies^[62-65] have revealed a statistically significant correlation between disease stage and the presence of tumor-associated genetic alterations in the blood of patients with several tumors. Currently, evaluation of response is only carried out as a standard procedure 3 months after initiating treatment, time by which the patient could be suffering side effects and toxicity without receiving any benefit, and without the opportunity of changing treatment. In this respect, lung cancer presents an added difficulty, namely the limited amount of tumor that can be obtained for molecular studies. Given the difficulty, the cost and the increased workload to obtain the sample, acquisition of tumor DNA is one of the limiting factors for patient selection. The availability of non-invasive tests that can detect molecular alterations in different genes will allow the study of biomarkers in all patients, thus overcoming these limitations and thereby radically changing clinical practice and increasing the treatment options for our patients.

Over the last decade, lung cancer treatment has evolved from administration of chemotherapy yielding poor outcomes, to biomarker assessments, such as EGFR, ALK or ROS1, with remarked improvements in OS^[66]. So, time has changed and personalized medicine has become a reality. However, to fully enable personalized medicine it is desirable to have an easily accessible, minimally invasive way to follow the molecular profile of a patient's tumor longitudinally. The peripheral blood of a cancer patient is a pool of cells or cell products derived from the primary tumor or from the metastatic sites. The analysis of these blood components provides a mean for minimally invasive molecular diagnostics, overcoming limitations of tissue acquisition, which is not always possible and satisfactory in NSCLC patients.

Technical developments achieved during the last years allow the identification and characterization of CTCs, cfDNA, exosomes and, recently, tumor-educated platelets, all known to play a main role in the carcinogenesis, relapse and progression of cancer. However, some of these analytical platforms typically have a suboptimal sensitivity^[67].

On the other hand, PET/CT and SUV assessments are widely criticized for being insufficient and imprecise, however to date no methods for reanalysis have been developed that also interact with tumor molecular status. Moreover, the combined, parallel histological study of tumor biopsies, with data regarding regression to treatment and which attempts to investigate cisplatin resistance mechanisms, may contribute to the design and use of new drugs. The liquid biopsy study using different techniques and procedures, and their correlation with histological response, the PET images and

functional radiological studies could prove as a valuable tool for the development of future clinical trials.

At a time when increasingly more new drugs are appearing, and with the unravelling of more complex molecular mechanisms, it is ever more necessary to translate these advances to the patient's bedside.

However, such advances are of little use if the chosen target population is not homogeneous, or is insufficiently or incorrectly assessed, or the research is so far removed from the patient that it will probably never contribute to improving a health problem.

Several studies, some of them analyzed in this editorial, have tried to focus on its role for screening and early detection of cancer, estimation of risk for metastatic relapse, identification of therapeutic targets and resistance mechanisms, and real-time monitoring of patients^[68]. As cancer is known a complex and dynamic disease, future prospective trials are needed to demonstrate its clinical utility. All these different blood products might contribute to an improved survival of cancer patients.

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