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REVIEW

Circulating nucleic acids in plasma and serum (CNAPS): applications in oncology

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Abstract: The presence of small amounts of circulating nucleic acids in plasma and serum (CNAPS) is not a new finding. The verification that such amounts are significantly increased in cancer patients, and that CNAPS might carry a variety of genetic and epigenetic alterations related to cancer development and progression, has aroused great interest in the scientific community in the last decades. Such alterations potentially reflect changes that occur during carcinogenesis, and include DNA mutations, loss of heterozygosity, viral genomic integration, disruption of microRNA, hypermethylation of tumor suppressor genes, and changes in the mitochondrial DNA. These findings have led to many efforts toward the implementation of new clinical biomarkers based on CNAPS analysis. In the present article, we review the main findings related to the utility of CNAPS analysis for early diagnosis, prognosis, and monitoring of cancer, most of which appear promising. However, due to the lack of harmonization of laboratory techniques, the heterogeneity of disease progression, and the small number of recruited patients in most of those studies, there has been a poor translation of basic research into clinical practice. In addition, many aspects remain unknown, such as the release mechanisms of cell-free nucleic acids, their biological function, and the way by which they circulate in the bloodstream. It is therefore expected that in the coming years, an improved understanding of the relationship between CNAPS and the molecular biology of cancer will lead to better diagnosis, management, and treatment.

Keywords: plasma, cancer, clinical tool, microRNA, heterozygosity, tumor suppressor genes, viral genomic integration, biomarkers

Introduction

According to the World Health Organization, cancer mortality will increase by 45% from 2007 to 2030 due to the demographic increase and population aging.[1] For that reason, many efforts have been made to find sensitive and specific biomarkers for early diagnosis, prognosis, and management of patients during treatment and follow-up.

A variety of tumors secrete proteins into the bloodstream that are routinely used as tumor markers in clinical practice, such as the prostate-specific antigen in prostate cancer, the alpha-fetoprotein in hepatocarcinoma, the carcinoembryonic antigen (CEA) in colon cancer, the cancer antigen (CA) 15.3 in breast cancer, and the CA19.9 in pancreatobiliary tumors.[2] However, those techniques might lack sensitivity in nonsecretory tumors, and might give positive results due to inflammatory processes or benign illnesses, limiting their specificity. In addition, there are no known serum markers for most tumors, which highlights the need for extensive study of the biology of tumors to propose new clinical tools. Indeed, it is expected that an improvement in

the knowledge of the molecular biology of cancer will lead to earlier diagnosis and more effective treatments.

Cancer is produced and progresses as a consequence of complex and gradual processes, in which a variety of genetic and epigenetic alterations are involved (eg, mutations, hypermethylations), and which mainly result in uncontrolled cell growth. Those molecular alterations can be found in the bloodstream,[3] which suggests that we could find understanding of the molecular biology of a specific cancer in plasma (Figure 1). This was the start point for the study of the so-called cell-free nucleic acids in plasma and serum (CNAPS) as cancer biomarkers.

Although in the last decades the CNAPS concept has generated a considerable interest in the research community, the concept was first suggested many years before this. Specifically, Mandel and Métais[4] first reported the existence of cell-free DNA (cfDNA) in plasma in the mid-twentieth century. However, their study had scarce impact until 30 years later, when Leon et al[5] demonstrated that cancer patients had higher cfDNA levels than normal people and that patients with persistently high levels of cfDNA after treatment had a worse prognosis than those in whom cfDNA levels decreased.

Some years later, Stroun et al[6] suggested that at least some of the cfDNA in serum or plasma was derived from the primary tumor, but this hypothesis was only confirmed later by two nearly parallel studies that described the presence of *KRAS* mutations in plasma from patients with pancreas neoplasm[7] and acute myelogenous leukemia.[8] Those discoveries were the milestones that opened new pathways for cancer biology research and the search for new clinical tools.

The term "CNAPS" refers to different types of cell-free nucleic acids (cfNAs), such as genomic DNA (gDNA), mitochondrial DNA (mitDNA), viral DNA and RNA, messenger (m)RNA, and microRNA (miRNA), which have recently been reported to be present in plasma.[3,9–13]

In spite of the increasing number of studies focused on CNAPS, and of the technological improvements, nowadays, some important aspects of CNAPS biology remain unknown, such as their release mechanisms,[14] method of circulation,[15–18] and biological role in cancer progression.[19] Limiting factors in the study of CNAPS have mainly been the lack of harmonization of laboratory techniques, the heterogeneity of the disease progression, and the small number of recruited patients in most of studies, which is for some authors[9] an example of poor translation of basic research into clinical practice.

Circulating cfDNA

Biology of cfDNA

It has been reported that cfDNA is a double-stranded molecule of low-molecular weight that is fragmented into short (70–200 base pairs) and long sections (up to 21 kilobases).[20] The analysis of those fragments has deepened understanding

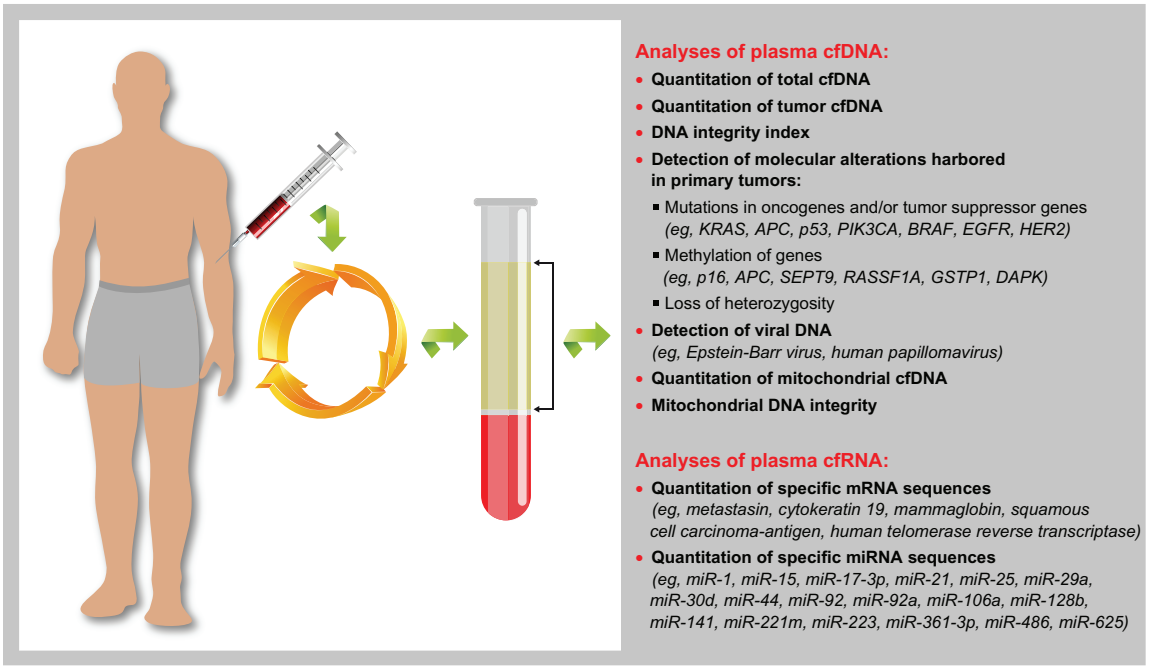

Figure 1 Graphic representation of the analyses of cell-free nucleic acid (cfNA) circulating in plasma that have been tested in cancer patients and that might serve as clinical markers.
Abbreviations: mRNA, messenger RNA; cfDNA, cell-free DNA; cfRNA, cell-free RNA; miRNA, micro RNA.

of their origin.[21] For some researchers, the main source of cfDNA has been necrosis,[22,23] while others have proposed apoptosis in view of the size of the DNA fragments detected in plasma.[20] In addition, several studies have suggested the active release of cfDNA by cells,[14,24] and specifically by lymphocytes.[25] Therefore, two possible sources of cfDNA, non-mutually exclusive, have been considered: passive release through cell death and active release by cell secretion. Nonetheless, recent studies using genome-wide sequencing of plasma DNA have demonstrated that such DNA contains representation of the entire tumor genome and reflects the clonal genomic evolution of tumors.[26]

Theoretically, CNAPS would be rapidly degraded in the bloodstream by nucleases; it has even been proven that mutated cfDNA degrades faster than non-mutated cfDNA.[27,28] However, the enzymatic action might be limited because at least part of cfDNA appears to be protected by being complexed or particulate with special protective characteristics against enzymatic degradation.[29–31] A decreased activity of DNase has also been observed in plasma from cancer patients, which might be another reason for the high levels of cfDNA found in plasma.[31]

Quantitation of cfDNA in plasma and serum

Many studies have confirmed the early findings of Leon et al.[3,9] In fact, high concentrations of cfDNA have been reported in plasma and serum from patients with various cancers, such as cancer of the colon,[33] lung,[34–36] breast,[37,38] stomach,[39] and esophagus.[40] The clinical value of such quantitation for differential diagnosis was suggested in a large study by Shapiro et al,[41] who showed that patients with benign gastrointestinal diseases had a lower mean concentration of plasma cfDNA (118 ng/mL) than cancer patients (412 ng/mL).

Several studies have proposed the use of the quantitation of cfDNA in plasma as a method for screening colon,[33] breast,[38] and lung cancers[36,42] among others. In colon cancer patients, such a method has showed an even better sensitivity than CEA quantitation.[33] In breast cancer patients, plasma levels of cfDNA have also been related to clinicopathological variables such as size, tumor stage, lymphadenopathies, human epidermal growth factor receptor 2 (HER2)/neu level and state.[38] In lung cancer, it has even been suggested that the concentration of circulating DNA might be a risk factor for the presence of the illness and a prognostic index during follow-up.[36]

However, in extensive revisions, the use of quantitation of cfDNA in plasma as a unique marker has been questioned[9] and has even been proposed as inadvisable in lung cancer[43] and associated with CA125 in ovarian cancer.[44] In addition, it is remarkable that overlapping concentrations of cfDNA are found in healthy individuals under physiological stress (eg, physical exercise) or in patients affected by other pathological processes, such as, inflammation, trauma, or sepsis.[3,45–47]

The validation of the clinical utility of methods for detection and quantitation of plasma cfDNA has probably failed because of technical limitations, particularly those related to sensitivity and specificity. There have been some attempts to establish reference values for different types of cancer;[48] however, the lack of harmonization in the laboratory techniques (quantitative polymerase chain reaction [PCR], spectrophotometry, fluorimetry, etc) and the low number and heterogeneity of patients enrolled in each study, have prevented the achievement of suitable statistical power and the establishment of reference patterns.[3,9,10]

Although, theoretically, the levels of cfDNA in plasma might be affected by several clinicopathological features such as tumor size, tumor stage, or metastasis,[9,38] no direct relationship between these features has been proven, as Lecomte et al discussed in their review focused on colorectal cancer.[49] García-Olmo et al have conducted studies in animal models to deepen understanding of cfDNA kinetics and have repeatedly shown that non-mutated DNA levels are not significantly related to tumor size or metastasis.[50,51] In fact, they found that large amounts of non-tumor DNA are released during tumor progression and, in particular, at the early stages, suggesting that there is active interaction between tumor and non-tumor cells.[50]

It is probable that the quantitation of cfDNA in plasma will be of most clinical value during disease monitoring.[42] It has been reported that plasma levels decreased in cancer patients after surgical treatments[33,35,37,39,40] and/or chemoradiotherapy,[52] sometimes reaching levels similar to those measured before treatment.[35,39,40] In addition, patients who maintained high levels of cfDNA in plasma either did not respond to the treatment or had a high risk of relapse.[5,33–37,53]

Circulating nucleosomes, as degradation products of necrotic tumor cells in the bloodstream, have also been examined and quantified by enzyme-linked immunosorbent assay techniques.[16] The DNA of necrotic cells, after being phagocytosed by macrophages,[23] might be released to the bloodstream inside those structures, which protect it from enzymatic degradation. Nucleosomes have been found in healthy subjects and in patients with benign diseases,[15] thus, their value as a screening method appears to be limited; however, there is some evidence to support their utility

for cancer monitoring. Specifically, during chemotherapy treatments, an initial increase of the levels of nucleosomes has been observed in plasma, which might be related to apoptosis provoked by treatment, and a later decrease in patients who had a good response to the treatment.[15,54,55]

Molecular alterations of cfDNA

Most of the molecular alterations found in cfDNA circulating in plasma reflect the genetic and epigenetic changes found in primary tumors and, thus, the analysis of such tumor cfDNA might be valuable for tumor diagnosis and monitoring. Highly sensitive methods are required to detect those alterations among larger quantities of non-altered cfDNA molecules and, for this reason among others, extremely varied results have been reported.[3,20,56]

Following, we review the alterations in cfDNA most frequently found in plasma from cancer patients and tumor-bearing animals.

Integrity of the DNA strand

Using PCR, Wang et al[57] found that long DNA fragments, related to necrosis phenomena, could be distinguished from shorter fragments, produced by physiological apoptosis phenomena. These findings gave rise to the so-called integrity index, based on the ratio between long and short cfDNA fragments. The integrity index was established based on gynecologic and breast cancers,[57] with the results obtained from 61 patients compared with those from 65 patients without neoplastic disease. It was found that the area under the curve for the DNA integrity index was 0.911 in cancer patients in relation to patients without cancer, with 100% sensitivity and 62% specificity. The authors suggested that the integrity index provides a simple and inexpensive way to detect cancer.[57]

A number of transposable elements of the genome, such as ALU and LINE1 sequences, can be easily detected and have been associated with tumor necrosis. Specifically, ALU sequences are short elements (typically 300 nucleotides in length) that account for more than 10% of the human genome. Umetani et al developed a method to measure the integrity of cfDNA in serum using quantitative PCR for ALU repeats, and suggested that the integrity index is increased at the early stages of the disease and a promising molecular biomarker for detecting colorectal and breast cancer.[58,59] Moreover, it might serve as a sensitive method to detect nodal metastases in the early stages, having an even higher predictive value than the clinicopathological variables commonly used, such as tumor size and stage.[59]

In a recent study in patients with hepatitis B virus-related hepatocellular carcinoma, the integrity index was associated with tumor size, TNM Classification of Malignant Tumors (TMN) stage, and nodal metastasis, and showed a more discriminatory power than total cfDNA concentration.[60]

Further, in other recent studies, the clinical value of the integrity index has been demonstrated, not only for the diagnosis but also the monitoring of the disease. For example, it has been found that the integrity index decreases in parallel to the response to surgical treatments for head and neck tumors[61] and melanoma,[62] to chemotherapy in leukemia,[63] to radiotherapy in nasopharyngeal tumors,[64] and to chemo-radiotherapy in colorectal cancer.[52] Moreover, it has been reported that patients with high concentrations of integrity DNA had shorter disease-free intervals.[64]

Alterations in the integrity of DNA have also been detected in other organic liquids, such as urine, and can be used in the early diagnosis of bladder cancer without cytology.[65] Some authors have emphasized that the integrity index meets many of the requirements of a universal biomarker;[10] however, other studies have not supported this idea.[66]

Gene mutations

As previously mentioned, many studies have shown that gene mutations found in primary tumors can be also detected in cfDNA circulating in plasma. The literature reports a wide percentage range of patients in whose plasma such mutations have been detected. The differences between studies might be due to technical reasons, since sensitive techniques are needed to detect very small amounts of mutated DNA among larger quantities of un-mutated DNA, which has a dilution effect.

The gene that has most frequently been examined is the *KRAS* oncogene, which is mutated in variable rates in high-incidence tumors, such as colon, pancreas, lung and thyroid tumors,[9] and has intrinsic characteristics that make it useful as a marker.[49] In fact, the prevalence of *KRAS* mutations is near 50%. Such mutations occur in the early stages of carcinogenesis and are mostly produced at specific sites (particularly in codon 12), which facilitate its detection using PCR-based techniques.

With respect to colorectal cancer, *KRAS* mutations have been found in 40%–50% of patients, and have also been detected in the plasma or serum of 25%–30% of patients.[49] Kopreski et al[67] tried to correlate the detection of *KRAS* mutations in plasma with clinicopathological findings detected by colonoscopy. They enrolled into their study one of the largest series of colorectal cancer patients that has ever been enrolled into similar studies, and detected *KRAS* mutations in plasma

from 83% of patients whose tumors had such mutations.[67] Moreover, they also found that some patients with apparently normal colonoscopy had *KRAS* mutations in plasma, which might be related to the presence of precancer lesions, thus suggesting the technique's suitability for early diagnosis and screening.[67]

In a review by Sorenson,[68] the author defended the detection of *KRAS* mutations in plasma as a specific marker for gastrointestinal tumors, with 2.5% false positives, although the review of studies showed that the concordance with *KRAS* plasmatic mutations with respect to primary tumor was only 50%. It has also been reported that other mutations, different from those in primary tumors, can be detected in plasma from cancer patients, which might be due to clone heterogeneity in tumors.[69]

In patients with pancreas cancer, it has been shown that detecting *KRAS* mutations in plasma is useful for early diagnosis as a complementary marker together with other serum markers (eg, CA19.9).[70] Other researchers have also used it for disease monitoring.[71]

In a prospective study conducted by Gormally et al[72] of a healthy population, *KRAS* mutations were detected in plasma from 1.2% subjects and p53 mutations in 3.6%. The authors suggested that *KRAS* mutations are detectable in plasma before the diagnosis of bladder cancer. In contrast, *KRAS* mutations have been detected by other researchers in up to 30% of healthy individuals;[9] this has been interpreted as a limitation of the diagnosis and screening value.

With respect to mutations in other genes, Chen et al[73] used an ultrasensitive technique to detect p53 mutations in plasma from patients with Stage II and III breast cancer, and their results showed a correlation between such detection and the clinical course of the disease after therapy.

The value of detection of tumor mutations in plasma for monitoring the response to treatment has been analyzed in several studies. Diehl et al[74] showed that the detection and quantification of mutated cfDNA in plasma from colon cancer patients undergoing surgery and chemotherapy were more useful for monitoring than the quantitation of CEA in serum. In addition, a recent study has shown that the use of panels with the most frequent mutations in colorectal cancer (*APC, KRAS, TP53, PIK3CA*, and *BRAF*) was more useful than using CEA and CA19.9 levels.[75] Further, it has been suggested that, in the postoperative period, the levels of mutated *KRAS* in plasma are a more powerful predictor of recurrence than Dukes stage.[76]

In breast cancer patients, the presence of amplified *HER2* has been demonstrated in circulating cfDNA during follow-up.[77] This led the researchers to propose such amplification as a marker for prognosis and response to treatment with monoclonal antibodies, such as trastuzumab.[77] A recent study has provided proof of the concept that tumor cfDNA circulating in plasma represents a highly sensitive biomarker of tumor burden in metastatic breast cancer.[78] Specifically, the researchers developed new methods to identify somatic genomic alterations (point mutations, structural variants) and designed personalized assays to quantify tumor cfDNA circulating in plasma from 30 patients. They found that levels of tumor cfDNA showed a greater dynamic range and greater correlation with changes in tumor burden than did CA15-3 or circulating tumor cells.[78]

Many chemotherapeutic agents act on pathways in which *KRAS, BRAF, EGFR*, or *p53* are involved.[79,80] For this reason, in many cases, it is important to know the mutation status for predicting the response to treatment and monitoring the disease. The analysis of plasma offers a noninvasive and quick way to find out this information and, in this sense, it has been reported that detection of *EGFR* mutations in plasma might be useful to predict disease progression, disease-free intervals, and drug resistances in patients with lung cancer.[79–81] Moreover, it has recently been reported that sequencing of cancer exomes in serial plasma samples might be useful to track genomic evolution of metastatic cancers in response to therapy.[26] The researchers described a noninvasive approach for characterizing cancer exomes in plasma that might enable detailed and comprehensive evaluation of clonal genomic evolution associated with treatment response and resistance.[26]

Summarizing, patients whose tumors have specific mutations might be monitored by analyzing the tumor cfDNA in their plasma samples.[10,26,78]

Gene hypermethylations

Some tumors are related to specific epigenetic alterations (eg, methylation), leading to changes in the expression of promoters of suppressor genes, which results in their silencing. Such alterations occur early in tumorigenesis and in DNA fragments that are rich in cytosine and guanine (CpG islands). The first observations were reported in 1999 by Esteller et al[82] in non-small cell lung carcinoma; now, the detection of these alterations represents one of the most promising advances in cancer diagnosis.

Epigenetic alterations are not tumor specific; moreover, there are some genes that are frequently hypermethylated and silenced in different types of tumors. In fact, many studies have analyzed panels of genes to increase sensitivity.[83–85]

Thus, it is essential to select accurately the genes to analyze for each type of cancer to improve the sensitivity of the analyses. Some of the most common aberrant methylations affect the *p16* tumor suppressor gene and have been found in patients with liver, lung, and breast tumors.[82,86,87] Other suppressor genes frequently hypermethylated are *SEPT9*, *RASSF1A*, *GSTP1*, and *DAPK*, among others.

The clinical value of the detection of hypermethylated genes in plasma has been shown in different types of cancer such as breast,[84,85] colon,[53] liver,[88] esophagus,[89] and urological tumors,[90] as well as in hematologic diseases.[91] It has been suggested that the methylated status of cfDNA circulating in plasma might be a tool for prognosis stratification and the prediction of the response to some chemotherapeutics agents,[8,9,92] disease-free interval, and risk of relapse.[53,88,89]

Hypermethylations have also been detected in cfDNA from other body fluids such as urine.[90] Specifically, the presence of tumor cfDNA has been reported in 70% of urine samples, suggesting its utility for the diagnosis and monitoring of patients with urologic tumors such as bladder, prostate and kidney cancers.[90]

Microsatellite alterations

"Microsatellites" are repeating sequences of one to six nucleotides that are scattered along the genome; their function is unknown. Such sequences serve to identify "loss of heterozygosity" (LOH), which is a frequent alteration of tumor DNA characterized by the loss of an allele when compared with matched normal DNA from the same individual. LOH indicates the absence of a functional tumor suppressor gene by deletion. In contrast, some tumors have abnormally long or short microsatellites as a result of a defective DNA repair process; this is termed "microsatellite instability" (MSI).

It has been reported that LOH and MSI can be detected in cfDNA circulating in plasma; however, discrepancies between tumor DNA and plasma cfDNA have been reported.[2] Although the detection of such alterations in plasma is more probable in advanced stages, some researchers have suggested a potential value for such detection at the diagnosis stage of breast[93] and ovarian cancers,[44] which may have an even higher sensitivity than the quantitation of cfDNA.[44]

To increase the sensitivity of this kind of marker, they have been tested in combination with several other plasma markers, such as methylations and prostate-specific antigen in prostate cancer.[94] In addition, panels of microsatellites are often used to improve sensitivity.[95,96] For example, in a recent prospective study in breast cancer patients, LOH was determined by PCR-based microsatellite analysis using a panel of eight polymorphic markers.[96] The researchers found that LOH at those markers was significantly correlated with tumor stage, tumor size, lymph node metastasis, positive progesterone, and HER2 status.[96] Moreover, LOH at a marker mapping to *cyclin D2* correlated with shorter overall survival. Thus, the researchers concluded that the improved detection of LOH on cfDNA provides important information on DNA losses of tumor suppressor genes (*TIG1, PTEN*, *cyclin D2*, *RB1*, and *BRCA1*) in breast cancer. In particular, loss of the *cyclin D2* gene may become an important prognostic marker easily detectable in the peripheral blood.[96]

Prior to that interesting study, it was reported that the assessment of microsatellite status in plasma might be a useful predictive tool for prognosis in breast carcinoma,[97] to monitor the response to surgical treatment; in oral squamous cell carcinoma;[95] and to biochemotherapy in metastatic melanoma.[98]

Viral DNA

Viruses are the main etiologic factors of a number of tumors and can be detected in plasma by PCR-based techniques. In fact, the presence of cfDNA of Epstein–Barr virus (EBV) has been demonstrated in nasopharyngeal cancers, Hodgkin's disease, and Burkitt lymphoma; human papillomavirus in cervical tumors; and hepatitis B virus in hepatocellular tumors, certain lymphomas, and gastric cancers.[3,10,12,99–102]

The presence of EBV sequences in plasma has been the focus of many studies, perhaps due to the high incidence of nasopharyngeal carcinoma in Asian countries. It has been shown that the detection of EBV in plasma is a powerful diagnostic tool and its quantitation might have prognostic value.[103] In addition, detection of EBV in plasma has also been associated with response to radiotherapy, disease recurrence, and survival.[12]

mitDNA

Although the study of the genetic and epigenetic alterations of gDNA is the cornerstone of cancer research, the discovery of specific alterations in mitDNA in cancer patients[104] has opened new routes in the search for clinical tools. The detection of aberrant changes in mitDNA is becoming an important tool for the early diagnosis of cancer, which is in part due to the fact that the analysis of mitDNA has some advantages over that of gDNA.[11] These advantages can be summarized as follows.

- The mitochondrial genome is shorter and more simply organized than nuclear DNA. These unique properties

make the screening of a mitochondrial genome much easier and more cost-effective.

- mitDNA's high number of copies, in comparison to nuclear DNA, make it a much more sensitive method.
- mitDNA fragments have been detected in different body fluids from cancer patients at early stages, such as in the blood,[104,105] saliva,[106] urine,[107] and sputum.

The alteration of mitDNA as a response to adaptation changes was first described in the early twentieth century, when it was termed the "Warburg effect."[108] Alterations in mitDNA have been found in the plasma of healthy individuals[109] and have also be related to tumor development and progression.[110] The alterations, which are point mutations, deletions, insertions, and quantitative changes,[11] have been detected in a wide range of tumors, such as breast, colon, liver, head, and neck and lung.[111] It is important to highlight that the identification of mutations in a concrete region typical to many tumors is referred to as the "D-loop," which may have diagnostic value.[104]

The presence of mitDNA mutations has been reported in plasma and serum samples from patients with hepatocellular, pancreatic, prostatic, colorectal, and esophageal cancers, among others.[112–116] Moreover, several studies have suggested that quantitation of mitochondrial cfDNA in plasma might serve as a clinical tool,[117,118] and may even have a higher diagnostic value than gDNA in some cases.[118] Specifically, Kohler et al[117] compared the levels of gDNA and mitDNA in plasma from patients with benign and malignant breast tumors with those from healthy controls. They concluded that both nuclear and mitochondrial cfDNA have potential as biomarkers in breast tumor management; however, the nuclear cfDNA showed greater sensitivity and specificity. Zachariah et al[118] conducted a study in 104 women with ovarian cancer, benign tumors, and endometriosis, and compared the levels of gDNA and mitDNA in plasma in these patients. Patients with epithelial ovarian cancer had significantly higher amounts of nuclear and mitochondrial cfDNA than the other women, but the levels of cfDNA in plasma were related to neither pathological parameters nor CA125 levels. In addition, the researchers found that quantitation of mitDNA was a unique way to differentiate between patients with ovarian cancer and endometriosis.[118]

Other studies have used "mitochondrial DNA integrity," which has been defined as the ratio between long and short fragments of mitDNA.[119] It has been reported that such an index might differentiate between patients with urologic malignancies (renal, prostate, and bladder tumors) and healthy subjects, with a sensitivity of 84% and a specificity of 97%.[119]

Finally, it has been reported that some mitDNA polymorphisms are associated with cancer development, thus a genetic analysis of such polymorphisms could help to identify target populations to establish screening programs.[11]

Circulating cell-free RNA (cfRNA)

Biology of cfRNA

The origin of the cfRNA circulating in plasma, its role, and its release mechanisms are yet unknown. The existence of cfRNA in blood was reported many years ago when, in 1987, Wieczorek et al[120] found RNA in proteolipid complexes in the serum of cancer patients.[121] However, the potential clinical value of the detection of cfRNA in plasma did not attract the attention of researchers until 1999, when two parallel studies reported the detection of tyrosinase mRNA in patients with metastatic melanoma[122] and mRNA associated with EBV in patients with nasopharyngeal carcinoma.[123] Many studies have subsequently reported the presence of specific mRNA in plasma from patients with a variety of cancers including colon,[124–126] breast,[127,128] prostate,[129,130] melanoma,[122] lung,[131] and thyroid.[132]

It was theorized that the fragility of cfRNA in serum or plasma – due to the fast enzymatic degradation it undergoes, which is increased in cancer patients[133] – might make its detection difficult. However, it was found that the molecules are more stable than it was presumed,[134] possibly due to protection by vesicle-like structures.

The origin of cfRNA remains less clear than that of cfDNA.[21,24] It has been proposed that apoptosis might be involved in the release of cfRNA, and that its association with apoptotic bodies might explain the resistance to nucleases in blood.[31] However, other hypotheses for the circulation of cfRNA have also been put forward, such as it occurring within lipoprotein complexes[24] or being in other actively-released particles, such as exosomes.[121]

Ng et al[135] examined the particle-associated nature of circulating cfRNA by filtering plasma samples from healthy subjects and cancer patients through material with different pore sizes. They found greater amounts of particle-associated mRNA in cancer patients than in healthy subjects, suggesting that most of the cfRNA not associated with particles had degraded.[135]

Detection and quantitation of cfRNA in plasma and serum

The use of cfRNA as a biomarker has several advantages including the ease with which plasma or serum samples for testing can be obtained, which makes it feasible for the monitoring of metastatic disease and even for wide

screenings. However, RNA molecules are fragile, so high sensitivity techniques with simultaneous internal controls are necessary.[136]

One of the most analyzed mRNAs in plasma is that of the human transcriptase reverse telomerase (hTERT), which corresponds to a ribonucleoprotein involved in the repair and lengthening of telomeres in eukaryotic cells. This mRNA is overexpressed in a variety of tumors.[124,126,128,129,137–140] However, cfRNA is not specific to cancer patients and can also be found in healthy volunteers or in those suffering trauma.[46,141]

In breast cancer patients, using PCR-based techniques, hTERT mRNA has been detected in serum, even in patients with localized disease.[128] Silva et al[127] observed that analysis of specific mRNA epithelium (cytokeratin 19) in plasma, in combination with mammaglobin, facilitates the detection of a greater number of positive cases than does analysis of tumor cfDNA (73% versus [vs] 29%). Moreover, detection of epithelial mRNA has been found to be related to tumor size and proliferation rate.[127] In addition, El-Abd et al have suggested the utility of the detection of metastasin mRNA in serum as a survival marker, with high sensitivity (85%) and specificity (100%).[142]

In colon cancer patients, an adequate correlation between hTERT levels in plasma and tumor stage has been observed, which has led such a quantitation to be proposed as a tool for screening, monitoring,[137] and response to treatment.[140]

With respect to lung cancer, Miura et al[138] analyzed plasma samples from 112 patients and 80 healthy subjects to detect and quantify hTERT and epidermal growth factor receptor (EGFR) mRNAs. They found that the sensitivity and specificity in lung cancer diagnosis were, respectively, 89% and 73% for hTERT mRNA, and 71% and 80% for EGFR mRNA. Moreover, they found that the number of copies of hTERT mRNA significantly decreased after surgical treatment.[138] These data led the researchers to suggest that hTERT mRNA, especially when combined with EGFR mRNA, may be an excellent biomarker for pulmonary malignancies to diagnose and assess clinical stage.[138] In addition, it has been suggested that the detection of heterogeneous nuclear ribonucleoprotein-B1 mRNA and HER2/neu-specific mRNA might have diagnostic value in lung cancer.[143]

In esophagus cancer patients, the detection of squamous cell carcinoma-antigen mRNA (SCC-Ag mRNA) by real-time PCR was shown to be the best predictive factor for recurrence in patients.[144]

In hepatocellular cancer, the detection of hTERT mRNA in serum has been suggested to be of diagnostic value, with a sensitivity of 88% and a specificity of 70%.[139] Moreover, the researchers reported a good correlation between the levels of hTERT mRNA in plasma and clinicopathological parameters, such as degree of differentiation.[139] However, other studies have shown no correlation with clinicopathological variables such as tumor size.[145]

cfRNA can be detected in other body fluids such as saliva and urine, and satisfactory results have been reported for its detection in these as a marker for the diagnosis of mouth and urological cancers, respectively.[136]

miRNA

The discovery of miRNA in 1993 was a milestone in cell biology research. From that moment, the number of identified miRNAs steadily increased and, to date, more than 900 human miRNAs have been discovered.

"miRNAs" are small noncoding molecules of 18–22 nucleotides that regulate gene expression, acting at a post-transcriptional level. It is known that a unique miRNA is able to regulate multiple different mRNAs, so that it might potentially alter the function of multiple channels.[13]

miRNAs have been demonstrated to play important roles in control of cell proliferation, cell differentiation, and apoptosis, thus their dysregulation contributes to tumor development. Moreover, it has been demonstrated that miRNAs can function as potential oncogenes or oncosuppressor genes, depending on the cellular context and on the target genes they regulate.[146] Aberrant expression of miRNAs has been associated with multiple types of cancer including colon,[147–150] lung,[151–154] breast,[155] prostate,[156] gastric,[157] and esophagus,[158] as well as lymphoma.[159]

miRNAs have been detected in the plasma and serum of animals and humans, and in both healthy subjects and cancer patients. The first evidence that miRNAs might serve as serological biomarkers of solid tumors was provided by Mitchell et al[156] and Chen et al.[147] miRNAs circulating in plasma have some advantages as clinical markers compared with other forms of cfRNA, since they have a remarkable resistance to endogenous and exogenous ribonuclease activity, extreme pH conditions, and freeze–thaw cycles, whereas synthetic miRNAs are promptly degraded.[160]

Circulating miRNAs have been found packaged into various membrane-bound vesicles such as exosomes,[18] microvesicles and apoptotic bodies (reviewed by Zandberga et al),[160] and also in lipoprotein complexes.[141] This is probably the main reason for the high resistance of plasma miRNAs, which makes their detection by PCR-based techniques easy. In addition, miRNAs are tissue specific, which allows

the development of panels to help in the diagnosis of tumors of unknown origin.[156,161,162] Moreover, they are not only detectable in the bloodstream but also in other body fluids.[18,163] Thus, the use of miRNAs as serological biomarkers is a very attractive option and research in this field continues to increase.

miR-21, probably the most analyzed miRNA, is often overexpressed in many types of tumors.[164] Nonetheless, a number of miRNAs have been demonstrated to have potential value as serological biomarkers in cancer, including miR-1, miR-10b, miR-17-92, miR-24, miR-92a, miR-122, miR-141, miR-155, miR-195, miR-221, and miR-375.[165]

The diagnostic value of miRNA panels appears to be superior to that of individual miRNAs.[160] Specific expression patterns of serum miRNAs have been identified for lung cancer, colorectal cancer, and diabetes, providing evidence that serum miRNAs contain fingerprints for a variety of diseases.[147]

It has been demonstrated that colorectal cancer patients have an miRNA serum profile significantly different to that of healthy subjects. Specifically, 69 miRNAs have been detected in sera from colorectal cancer patients but not in those from healthy subjects.[147] Moreover, colorectal cancer patients have been found to share a large number of serum miRNAs with lung cancer patients.[147] In addition, Ng et al[148] analyzed plasma samples from 90 colorectal cancer patients and 50 healthy subjects and found that miR-17-3p and miR-92 were overexpressed in patients, which suggested the potential diagnostic value of such plasma levels, with a sensitivity of 89% and specificity of 70%. Subsequent studies have supported Ng et al's findings, including that by Huang et al,[149] who showed that levels of miR-29a and miR-92a in plasma discriminate colorectal cancer, with 83% sensitivity and 85% specificity. In a recent revision, the prediction value of three miRNAs (miR-221, miR-141, and miR-29a) has been highlighted as 3 independent factors of wrong predictions in different clinical stages.[166]

The increased expression of miR-21, miR-106, and miR-15 has been observed in breast cancer patients compared with that observed in healthy subjects, in both tissues and serum, and those levels have been found associated with tumor stage and the presence of lymph node metastases.[155] With respect to lung cancer, Wang et al[152] and Liu et al[153] observed high levels of miR-21 in the plasma of cancer patients compared with that in healthy individuals. These levels were also associated with TNM stage and the presence of lymph node metastasis. Further, Roth et al[154] suggested that miR-361-3p and miR-625 might have a protective effect on the development of non-small cell lung cancer, while its quantification in serum might have the diagnostic potential to detect that cancer, particularly in smokers.

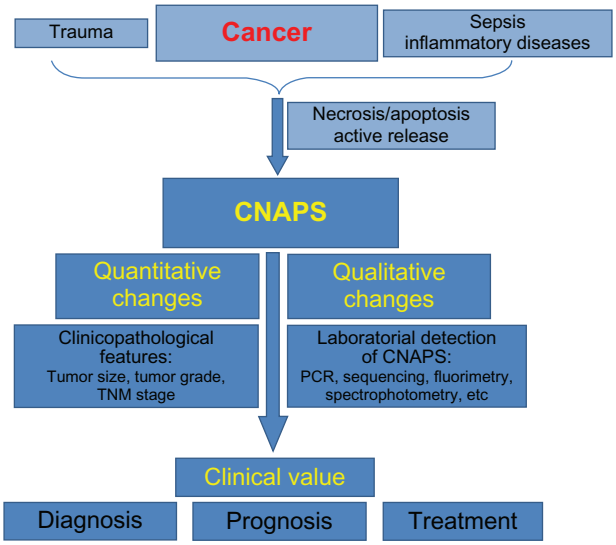

Figure 2 Schematic representation of the outline of the present review.
Abbreviations: CNAPS, cell-free nucleic acids in plasma and serum; PCR, polymerase chain reaction; TNM, TNM Classification of Malignant Tumors.

The most comprehensive search to date for circulating miRNAs with prognostic significance was performed by Hu et al,[151] who analyzed a large cohort of 303 patients and found that four plasma miRNAs (miR-486, miR-30d, miR-1, and miR-449) were independent predictors of average survival. In prostate cancer, Mitchell et al[156] were able to distinguish patients with cancer from healthy individuals by serum levels of miR-141. Finally, Lawrie et al demonstrated that serum levels of miR-21 were associated with the disease-free interval in patients with diffuse B-cell lymphoma.[159]

In future, the search for circulating miRNAs of clinical value is likely to be carried out at the same time as the study of many unknown aspects, such as the role of circulating miRNAs, the cell types that secrete them, and the regulation of this secretion.

Conclusion

The data reviewed in the present article suggest that the clinical value of cfNAs circulating in plasma is already more than a theoretical idea, since the characterization and the quantitation of such nucleic acids (NAs) have been shown to be complementary tools in the diagnosis, prognosis, and management of cancer patients (Figure 2). However, some important questions remain unanswered, largely because most studies have been underpowered as well as because there has been no adequate standardization of laboratory techniques,

which complicates the comparison of results from different groups.[3,9,10] Thus, it appears necessary to drive initiatives directed toward validating and verifying laboratory methods and procedures for required molecular tests before their use in clinical testing. For this, it might be useful to network, as has been demonstrated by successful previous initiatives.[167] Methods based on sequencing (genome-wide sequencing, exome sequencing, etc) appear to be the definitive tool for making the analysis of cfDNA for cancer monitoring useful, since it allows for personalized assays.[26,78]

However, plasma cfNAs have yet many enigmatic aspects. Elucidation of these might lead us to a revision in the way the pathobiology of cancer is conceived. It is likely that many molecular pathways are involved in the origin of such NAs, since various results point in non-exclusive different directions. It has been postulated that the cfNAs circulating in plasma are not merely biological waste, but that they may be directly involved in the development of metastases, possibly through transfection-like uptake by susceptible cells.[168] This hypothesis, the "genometastasis theory," is supported by strong evidence – namely, the observation that plasma from cancer patients can transfect and oncogenically transform cultured cells.[169,170]

Regardless, no conclusive explanation of the circulation mechanism of cfNAs yet exists. Several groups have suggested a relationship between the presence of cfNAs in plasma and the presence of exosomes.[121,171] Thus, it appears feasible that at least part of plasma cfNAs circulates within exosomes. This finding correlates with the idea that plasma cfNAs might have a role in tumor progression, since it has been proven that exosomes are able to transfer their RNA content to cells, and that this RNA can be functional in its new location.[18] It has even been demonstrated that miRNAs are transferred during immune synapsis and are able to modulate gene expression in recipient cells.[172] Moreover, cross-talk between tumor-derived exosomes and host cells, such as bone marrow progenitor cells, appears undeniable.[173] Thus, transfer of genetic material from exosomes to cells might be involved in the recruitment and metastatic conversion of host cells. Conversely, it might be possible to exploit this phenomenon for therapeutic purposes and, perhaps, it will be proper to focus the development of clinical tools on the detection and analysis of NAs containing microparticles. Certainly, at this moment, a robust body of research on exosomes and microvesicles exists and increased understanding of such particles in future will shed light on cancer pathobiology and offer promising perspectives on clinical tools.

Thus, although the origin and the circulation mechanism of plasma cfNAs remain unclear, there is strong evidence to suggest that such NAs will become useful biomarkers for the diagnosis and monitoring of cancer disease and that they might be essential to tumor development and progression.

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Disclosure

The authors declare no conflicts of interest in this work.

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