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Review

Clinical relevance of circulating molecules in cancer: focus on gastrointestinal stromal tumors

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Abstract: In recent years, growing research interest has focused on the so-called liquid biopsy. A simple blood test offers access to a plethora of information, which might be extremely helpful in understanding or characterizing specific diseases. Blood contains different molecules, of which circulating free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cells (CTCs) and extracellular vesicles (EVs) are the most relevant. Conceivably, these molecules have the potential for tumor diagnosis, monitoring tumor evolution, and evaluating treatment response and pharmacological resistance. This review aims to present a state-of-the-art of recent advances in circulating DNA and circulating RNA in gastrointestinal stromal tumors (GISTs). To date, progress in liquid biopsy has been scarce in GISTs due to several issues correlated with the nature of the pathology. Namely, heterogeneity in primary and secondary mutations in key driver genes has greatly slowed the development and application in GISTs, unlike in other tumor types in which liquid biopsy has already been translated into clinical practice. However, meaningful novel data have shown in recent years a significant clinical potential of ctDNA, CTCs, EVs and circulating RNA in GISTs.

Keywords: circulating tumor cell, ctDNA, CTCs, epigenetics, gastrointestinal stromal tumor, GIST, liquid biopsy, personalized medicine, precision medicine, soft tissue sarcoma

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Introduction

Technological advances, particularly next-generation sequencing (NGS), have paved the way to personalized medicine by drastically reducing the time and costs required to assess an individual's and disease's genetic make-up. Nowadays, it is indisputable that NGS technology provides the opportunity to look with unprecedented depth into biological samples, identifying low and ultralow frequency DNA variants.[1–3] One promising application of NGS technology is liquid biopsy for cancer detection and monitorization towards a personalized cancer-medicine strategy. In recent years, not surprisingly, we have witnessed a growing research interest in liquid biopsy. The term 'liquid biopsy', according to the *NCI Dictionary of Cancer Terms* (www.cancer.gov), is defined as 'a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood'. Indeed, a simple blood test offers access to a plethora of information, which might be helpful in understanding or characterizing a broad spectrum of diseases, including cancer.[4,5] Blood contains different molecules, including circulating free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating RNA (cRNA) and extracellular vesicles (EVs).[6–11] All these molecules together potentially permit the diagnosis of tumors, monitoring their evolution, and evaluating treatment response and drug resistance.[6,8,12–14] Consequently, liquid biopsy offers pivotal implications in clinical management, promising to revolutionize the standard management of oncological patients (Figure 1). Specifically, the variety of liquid biopsy applications includes:

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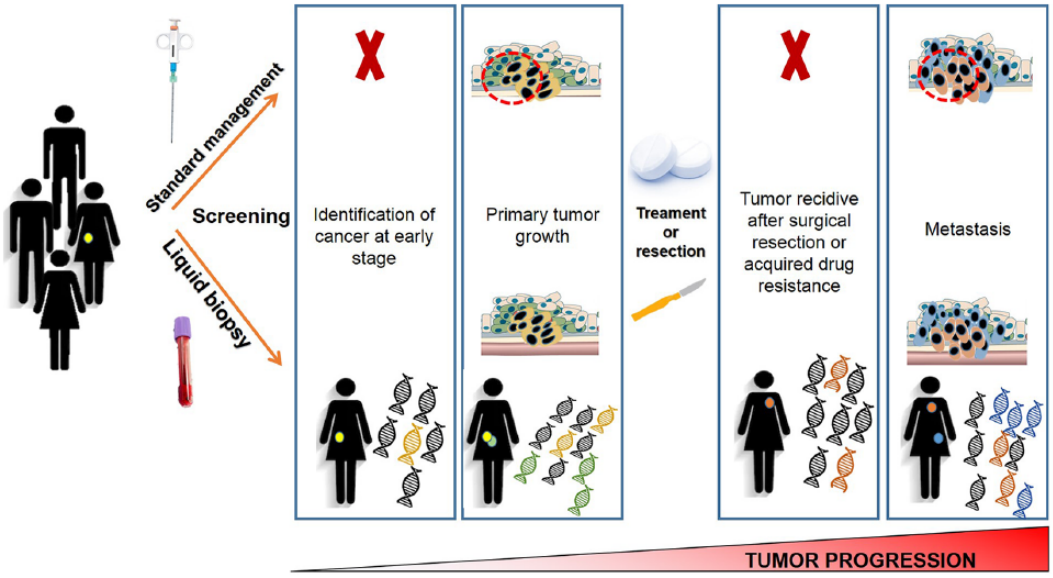

Figure 1. Patients' management: standard biopsy versus liquid biopsy. Potentially, a simple blood test may promote the identification of tumors at an early stage, in contrast with standard biopsy, which can be done only with advanced disease. Moreover, liquid biopsy has the advantage of providing a dynamic picture of the tumor, whereas standard biopsy may give only a static image, resulting from the small tumor tissue collected. Finally, liquid biopsy can be helpful to monitor the therapy response, due to the fact that it can detect novel resistance mutations which suggest the tumor is no longer responding to the treatment.

- Biological and clinical understanding of the disease
- Risk-based stratification of tumor patients
- Personalization of therapy
- Evaluation of clinical outcome, including therapeutic efficacy assessment.

cfDNA, ctDNA, CTCs, cRNA and EVs

cfDNA and ctDNA

Current evidence points to cfDNA being released during physiological cell functions and refers to DNA fragments outside of cells in different body fluids, including the plasma, serum, urine, and saliva.[15,16] The major part of extracellular DNA is adsorbed to the surface of leukocytes or erythrocytes (cell-bound DNA) in the bloodstream.[17,18] A portion can be identified in the plasma and it is known as cfDNA. cfDNA has a short half-life and is often heavily damaged, mainly due to its easy degradation by nucleases.[15,19–21] cfDNA also includes ctDNA, which is DNA-derived from tumors.[6] The exact mechanism through which ctDNA reaches body fluids is still unclear, although it has been proposed that apoptosis or necrosis of tumor cells, or active secretion from macrophages that have phagocytized necrotic cells, may have a prominent role in this process.[9,18,21] DNA concentration in plasma varies greatly from one individual to another; for example, the cfDNA concentration is lower in healthy people than in cancer patients (10–20 mg/ml *versus* >1000 mg/ml),[11,22,23] suggesting that the major contribution is given by ctDNA, while normal DNA only represents a small portion. As a result, ctDNA has emerged during the last decade as a novel and key source of information, profoundly diverse from tissue biopsy. Some key studies, across several cancer types, have also shown that mutations leading to treatment resistance can be detected in ctDNA several months before detection by imaging, suggesting its potential in monitoring drug response.[24,25] Finally, liquid biopsy offers repeatability due to its minimally invasive nature, which in turn leads to better acceptance by patients.[19]

CTCs

Recently, CTC analysis has become a significant field of study in biomedical research. In particular, CTC detection has emerged as an early marker of tumor recurrence, occurring before clinical symptoms manifestation, in various tumor types.[26,27] CTCs are tumor cells that may be released by early tumor lesions or metastases, generating expectations by the research

community for the development of a blood-based cancer test. However, CTCs detected in blood are usually in low numbers, being estimated that ~1–10 CTCs per ml of blood released by primary tumors or metastases may be detected in peripheral blood.[28–31] Therefore, the development of a reliable CTC-based test for early cancer detection or monitoring cancer progression remains challenging. In addition, CTCs are heterogeneous and may circulate as single cells or clusters of cells, making their use in the clinical setting even more complex. For example, it has been observed that CTC clusters may have a higher metastatic potential and a shorter half-life in circulation.[32,33] The majority of CTCs die in the bloodstream due to different causes, including physical and oxidative stress and paucity of growth factors and cytokines. However, the cancer cells that survive can exit the bloodstream and reach the surrounding tissues, where they start to divide and grow.[34,35]

cRNA

More recently, research in the liquid biopsy field has also focused on cRNAs, which includes mRNA and noncoding RNA (ncRNA). To date, it is well established that cRNAs are crucial mediators in cell-to-cell communication and in the regulation of gene expression and biological functions in recipient cells, thereby acting like hormones.[36,37] Similarly to cfDNA, cRNAs are highly vulnerable to degrading enzymes in the bloodstream, such as RNases. Given the critical role of these messengers, cRNA is preserved enclosed in EVs, including microvesicles, exosomes and apoptotic bodies, or complexed with specific RNA binding proteins, such as Argonaute 2 (AGO2), high-density lipoprotein and low-density lipoprotein.[7,38–41]

According to a length cut-off of 200 nucleotides (nts), ncRNAs encompass two super families: small ncRNAs and long ncRNAs (lncRNAs). Small ncRNAs comprise (1) microRNA (miRNAs) and small interfering RNAs (siRNAs), mediating RNA-silencing at the post-transcriptional level; (2) small nuclear RNAs (snRNAs), regulating splicing; (3) small nucleolar RNAs (snoRNAs), which may affect ribosomal RNA, transfer RNA, and snRNA processing; and (4) P-element-induced-wimpy testis (piwi)-interacting RNAs (piwiRNAs), which regulate chromatin modification and have transposon-silencing capabilities.[36] The lncRNA family comprises ncRNAs that are heterogeneous in both size, from 200 to 10,000 nts, and role. Specifically, lncRNAs may act as regulators of gene expression, as scaffolds for protein binding and as decoys for different RNA molecules, including miRNAs.[36,42–44] Additionally, deregulated lncRNA expression has been associated with the development of diseases, including cancer.[45–47] Among all these circulating ncRNAs, miRNAs are the most known and well-characterized. miRNAs, at approximately 20–22 nts in length, exert their action as modulators by binding specific seed sequences on the 3′UTR of specific target genes. It has been widely reported that circulating miRNAs are extraordinarily stable in body fluids[48] and the amount and composition of exosomal miRNAs differ between cancer patients and healthy controls, suggesting these miRNAs may represent potential non-invasive biomarkers.[49–51] The most recently discovered class are circular RNAs (circRNA), functioning as sponges for miRNAs or proteins.[52] circRNAs generally formed by the alternative splicing of pre-miRNAs, with 3′ and 5′ ends covalently linked,[53] are relatively abundant in exosomes and represent a new frontier in cancer research.[54–58]

EVs

EVs are cell-derived submicron membranous vesicles released into extracellular space.[59–61] They are small, lipid-bound particles packaging diverse nucleic acids and protein cargo, which are secreted from cells within normal and pathological conditions.[62] These can be potentially released in all body fluids, including saliva, blood, urine, breast milk and tears. Exosomes are the smallest vesicles, ranging in size from 30 to 100 nm and are generated by exocytosis of multivesicular bodies (MVBs) (Figure 2).[63] Microvesicles (MVs) are larger vesicles with a size spanning between 100 and 1000 nm, formed through a finely regulated budding/blebbing of the plasmatic membrane. MV production is generally low under physiological conditions, whereas tumors excrete them in a high constitutive manner.[63–68]

Taken together, ctDNA and cRNA offer the chance to gain a time-dynamic picture of the tumor, allowing the following of the eventual progression, pharmacological response or appearance of drug resistance.[13,69]

This review aims to present a state-of-the-art of the current progresses in ctDNA and RNA findings in GISTs.

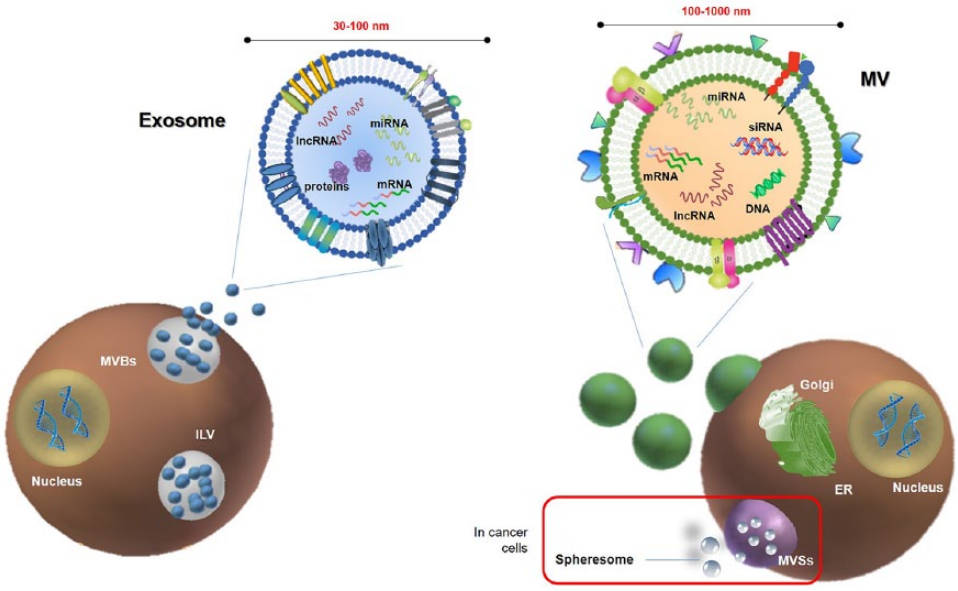

Figure 2. Schematic representation of extracellular vesicle (EV) release. Left panel: Exosomes (30–100 nm in size) are released in extracellular space from multivesicular bodies (MVBs) through exocytosis. MVBs contain various intraluminal vesicles (ILVs) which are generated by the inward budding of the endosome membrane. Exosome cargo may include different kind of RNAs, such as miRNA, lncRNAs, mRNAs, otherwise quickly degraded if free. Right panel: Microvesicles (100–1000 nm in size) originate through a finely regulated budding/blebbing of the plasmatic membrane involving the Golgi apparatus. According to the classical secretory pathway, vesicles with their protein cargo, are sorted and packed in the Golgi apparatus, and then transported to the plasma membrane. In cancer, it has been proposed that there is an additional mechanism of EV release. Specifically, cancer cells may produce multivesicular spheres (MVSs), which contain many spheresomes.

Table 1 summarizes the main features of cfDNA, ctDNA, CTCs, cRNA and EVs

Clinical epidemiology of GISTs

GISTs are rare sarcoma with an incidence in Europe of 15–20 cases per million per year. Although GISTs account for fewer than 1% of all gastrointestinal tumors, they constitute the most common sarcoma subtype.[70] *KIT* or *PDGFRA* gain-of-function mutations are the key drivers of this neoplasm and are found in approximately 85–90% of GISTs, whereas 10–15% do not present any alteration on these genes and are referred to as *KIT*/*PDGFRA* wild-type (WT) GISTs.[71–76] *KIT*/*PDGFRA* mutant GISTs include a plethora of different primary mutations in well-known regions. Primary mutations in *KIT* involve exons 9, 11, 13 or 17, whereas exons 12, 14 or 18 are the major hotspots in the *PDGFRA* gene. Single amino-acid substitutions, in-frame deletions or insertions are the predominant mutations types regardless of the gene and the exons. The development of tyrosine-kinase inhibitors (TKIs), such as first-line imatinib, revolutionized GIST management in the early 2000s through specific targeting of *KIT*/*PDGFRA* molecular drivers. However, the majority of GIST patients experience disease progression associated with the acquisition of secondary *KIT*/*PDGFRA* alterations; alternatively, the involvement of pharmacogenetic and epigenetic mechanisms on imatinib resistance has been investigated, without conclusive results.[75–80] To overcome the loss of imatinib response, the multikinase inhibitors sunitinib and regorafenib have been introduced successively as standard second- and third-line treatments, adding clinical benefits to GIST patients.[81–85] The emergence of new studies, together with the implementation of cutting-edge technologies in the field, highlights that the ever-increasing complexity in GIST molecular biology challenges the success of consecutive lines of treatment.[86] In the light of this complexity, liquid biopsy could emerge as a windfall in the near future. To date, progress in liquid biopsy in GISTs have been hampered mainly by the heterogeneity of primary and secondary mutations in *KIT* and *PDGFRA* receptor tyrosine kinases.[87,88] Indeed, this heterogeneity in mutational hotspots has slowed down

Table 1. Summary of the main features of circulating biomaterials.

Biomaterial	Type	Molecular size	Physiology	Analytical technique (I: isolation; A: analysis approaches)	Advantages	Disadvantages
cDNA	**cfDNA**	Highly fragmented; usually 166–320 bp in lenght	Released through necrosis, apoptosis, and active secretion. In healthy controls cfDNA levels are usually on average 30 ng/ml; in tumor patients cfDNA is usually 30% higher than healthy controls but can be up to 1000 ng/ml	**I:** Automated extraction method with MyOne Dynabeads;[20] polymer mediated enrichment or a phenol-chloroform based extraction procedure;[11] magnetic bead-based method **A:** NGS methods, ranging from WGS or WES to targeted sequencing of a limited gene panel	• Non-invasive; • Usable as prognostic and predictive biomarker; • Monitoring tumor progression	• Integrity of cfDNA may be compromised in the transportation, storage, and handling of samples; plasma cfDNA degrades by 30% for each year of storage;[21] • inability to specifically quantitate ctDNA fraction in cfDNA; • concentration of cfDNA did not seem to be associated with OS or PFS[11]
	ctDNA	Shorter fragment than cfDNA, highly fragmented DNA	0.01–90% of the total cfDNA;[11] secreted by tumor cells as a signaling molecule to drive tumor metastasis	**I:** ctDNA isolation follows the same approaches of cfDNA extraction; **A**: targeted approaches in which few tumor-specific mutations known from the primary tumor are used for monitoring residual disease (qRT-PCR, BEAMing, Safe-SeqS, CAPP-Seq, and TAm-Seq); genome-wide analysis for CNAs or point mutations by WGS or WES	• Monitoring therapy efficiency by detecting mutation-driven resistance; • Non-invasive	• Limited half-life: from 16 minutes to 2.5 hours;[11] The rate depends on the location, size, and vascularity of the tumor
CTCs		5–30 μm	EpCAM+/cytokeratin+/ DAPI+/CD45 cell[31,35]	**I:** Centrifugation or high-resolution imaging combined with immunocyto-fluorescent staining;[7] specific detection kit (i.e Cellsearch® system) **A:** CTCs can theoretically be characterized by all of the 'omic' assays that are usually performed on tissue biopsies. (i.e RNA-seq, CNAs, mutations' analysis, epigenetic changes evaluation)	• CTCs allow all the 'omic' analyses, as DNA sequence analysis, and study of mRNA and proteins expressed by tumor cells[35]	• Very rare (~1 CTC per ml of blood)

(Continued)

Table 1. (Continued)

Biomaterial	Type	Molecular size	Physiology	Analytical technique (I: isolation; A: analysis approaches)	Advantages	Disadvantages
cRNA	**ncRNA** in complex with specific RNA binding proteins	Sizes varies upon RNA types (i.e. miRNA: ~20–22 nt, lncRNA: >200 nt)	Released mostly by through active secretion (i.e. role of messenger between cells); cRNAs are highly vulnerable due to degrading enzymes in the bloodstream; preserved enclosed in EVs or complexed with specific RNA binding proteins	Specific kits (i.e. Mirvana Paris or Trizol LS); qRT-PCR, microarray, and NGS[58]	• Potential non-invasive diagnostic biomarkers; • More stable than other circulating molecules	• Difficult measurement due to the low concentration and high RNase activities in the extracellular space
EVs	**Exosomes**	30–100 nm	Released during reticulocyte differentiation as a consequence of multivesicular endosome fusion with the plasma membrane	**I:** Ultracentrifugation, Specific kit, novel high-resolution flow cytometry-based method. Electron microscopy, flow cytometry, DLS, and nanoparticle tracking analysis[61] **A:** analysis depend upon exosome cargo to evaluate (i.e. protein, RNA or DNA)	• Content of exosomes is a reflection of what the cell is experiencing; dynamic mediators of intercellular communication;[57] • Sensitive and non-invasive method, allowing the detection of tumors at an early stage; • Efficient tissue-specific, non-immunogenic carrier to deliver therapeutic drugs[68]	• Exosomes are potentially released by all the type of cells, including the nontumoral cells. • Some proteins are consistently associated with exosomes, not specific for cancer
	MVs	100–1000 nm	Budding/ blebbing of the plasmatic membrane, from lipid rafts	**I:** same methods used for exosomes **A:** same used for exosomes	• Similar to exosomes	• Similar to exosomes

CAPP-Seq: cancer personalized profiling by deep sequencing; CNA: copy number aberration; CTC: circulating tumor DNA; DLS: dynamic light scattering; EV, extracellular vesicle; GIST, gastrointestinal stromal tumor; MVs: microvesicles; NGS: next-generation sequencing; OS: overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; qRT-PCR: quantitative-real-time reverse transcription PCR; Safe-SeqS: safe-sequencing system; TAm-Seq: tagged-amplicon deep sequencing; WGS: whole genome sequencing; WES: whole exome sequencing.

liquid biopsy development and clinical use in GIST patients, unlike in other tumors such as lung cancer, in which it is being successfully implemented.[89] Given this assumption, in the following sections, we will analyze the most recent findings in liquid biopsy, considering both cDNA and cRNA in GISTs.

ctDNA and cfDNA in GISTs

The majority of reports are focused on primary and secondary *KIT* alterations, and aim to develop ctDNA as a novel biomarker to help clinicians in management of GIST patients. The first reported evidence for liquid biopsy in GISTs was presented at the 2013 ASCO Annual Meeting.[90] In this study, Demetri and colleagues analyzed both plasma and tissue DNA from a subgroup of GIST patients with advanced disease following imatinib and sunitinib failure, enrolled in the phase III GRID study. Specifically, the authors compared DNA mutational status (performed by Sanger sequencing) from archival tumor tissues with plasma samples (analyzed with bead emulsion amplification and magnetics; BEAMing). The analysis showed an 84% overall concordance in detection of primary *KIT* exon 9 and 11 mutations between tumor tissue and plasma. In particular, the concordance was 100% for *KIT* exon 9, but only 79% for *KIT* exon 11. Remarkably, BEAMing detects more frequently *KIT* secondary mutations in plasma (47%) compared with tumor tissue (12%). Overall, this represents the first report validating the feasibility of plasma-based ctDNA analysis in GISTs. In the same year, Maier and colleagues reported the results from patients enrolled in the NCT01462994 trial.[91] This study aimed to detect *KIT* and *PDGFRA* mutations in cfDNA in plasma from GIST patients with known activating *KIT*/*PDGFRA* mutations in tumor tissue, regardless of disease status and planned treatment.[92] Specifically, the authors evaluated 291 plasma samples from 38 GIST patients using 25 different allele-specific ligation (L)-polymerase chain reaction (PCR) assays covering common *KIT*/*PDGFRA* mutations. Mutations were detected in 15 patients, 9 having active disease (i.e. having at least one progressing lesion or responding to treatment) while the remaining 6 were without evidence of residual disease after surgery. Interestingly, the authors observed dynamic changes in mutant/wt allele ratios correlated with the course of the disease.[92] For instance, they showed a decrease in mutant cfDNA or negativization in patients responding to TKIs. Of note, all six patients with no evidence of disease had a high ($n = 5$) or moderated ($n = 1$) risk of relapse (Miettinen criteria), emphasizing that mutant cfDNA might also serve as tumor-specific biomarkers for the early prediction of recurrence in localized GISTs. Despite these promising data, reports on liquid biopsy in GISTs are still relatively limited (Table 2).

Following the work by Maier and colleagues, Yoo and collaborators assessed circulating biomarkers in TKI-refractory GIST patients recruited in a single-arm phase II trial using dovitinib.[93] BEAMing analysis of ctDNA identified primary kinase mutations in 16.7% of the patients; these mutations were 100% concordant with the results observed in the corresponding tumor tissue.[51] The detection of primary mutations was relatively low, compared with secondary *KIT*/*PDGFRA* mutations. This result is not surprising considering that BEAMing better detects predesigned point mutations, common as secondary mutations, rather than the complex *KIT* primary mutations in exon 11. Subsequently, Bauer and collaborators reported additional results on liquid biopsy in GISTs at the 2015 ASCO Annual Meeting. In particular, they prospectively collected 30 plasma and 15 matched tumor samples from 22 metastatic GIST patients.[94] Using a custom-designed targeted sequencing panel in an Illumina Miseq platform, they detected a total of 87 nonsynonymous *KIT* mutations in plasma samples. Primary mutations, all matching tumor analysis, were identified in 41% of GIST patients; resistance mutations were observed in 86% of GIST patients, although they were also observed in patients responding to imatinib.[94] Recently, Kang and collaborators, using NGS, provided more data from plasma samples through the monitorization of three GIST patients under TKI treatment. The authors analyzed tumor mutational status in baseline tumor biopsies and plasma samples collected during the follow up.[95] Additional mutations in plasma emerged in those patients who had a partial response or progressive disease, whereas they kept detecting only the primary mutation in the patient with stable disease.[95] The study had important limitations, such as the lack of plasma samples with matched biopsies and the absence of NGS data confirmation with a different technique. Particularly, considering that *KIT* and *PDGFRA* mutations are mutually exclusive, we may assume that the presence of a secondary *PDGFRA* mutation on a patient harboring

Table 2. Summary of the studies performed in GIST patients and included in the review.

Author, year	Aim	Patients		Technique	Result
		N **of tumor samples**	***N*** **of plasma samples**		
Demetri and colleagues[90]	To consider circulating DNA in plasma as a source of tumor DNA.	102 tissue	163	Sanger sequencing (tissue) and BEAMing (plasma)	Demonstrated utility of plasma-based circulating DNA analysis of target oncogenes.
Maier and colleagues[92]	To detect tumor DNA carrying *KIT* or *PDGFRA* mutation in plasma and correlate its discovery with the disease clinical course.	FFPE from 38 GISTs	291 (from 38 GISTs)	Allele-specific L-PCR assay	- Confirmed presence of *KIT*/*PDGFRA* mutant cfDNA. - The amount of mutant cfDNA correlates with the disease clinical course, being significantly higher in patients with active disease compared with those in complete remission.
Yoo and colleagues[93]	To assess the relevance of soluble serum proteins and ctDNA as biomarkers for TKI-refractory GISTs.	Archival tissue from 28 GIST	58 (from 30 GISTs: $n = 30$ day 1 of cycle 1; $n = 28$ day 1 of cycle 2)	BEAMing	Demonstrated usefulness of serum ctDNA for the identification of TKI-resistant mutations.
Bauer and colleagues[94]	To evaluate plasma sequencing to detect or monitor the spectrum of resistance mutations in GISTs.	Tumor tissue from 15 GISTs	30 (from 22 GISTs)	Illumina MiSeq platform	Showed that plasma sequencing detects a multitude of resistance mutation in *KIT* and other genes.
Kang and colleagues[95]	To analyze ctDNA from the plasma of GIST patients on TKI therapy.	Tumor tissue from 3 GISTs	Not specified (from 3 GISTs)	NGS	- Demonstrated detection of primary and secondary mutations in ctDNA. - Resistant mutations in ctDNA may represent early biomarkers for treatment response.
Wada and colleagues[96]	To investigate if secondary *KIT* mutations can be detected in ctDNA.	Primary tumor and imatinib-resistant lesion from 4 GISTs	8 (from 4 GISTs: samples taken before and after the treatment of imatinib-resistant lesions with sunitinib)	NGS	- Confirmed detection of *KIT* secondary mutation in ctDNA. - Secondary mutation in plasma were the same identified in imatinib-resistant tumor tissue. - The fraction of ctDNA changed along with tumor status.
Kang and colleagues[97]	To validate the use of ctDNA as a biomarker for determining *KIT* and *PDGFRA* mutations.	FFPE from 25 GISTs	25 (from 25 GISTs, taken before surgery)	Sanger sequencing (tissue), NGS (tissue and plasma)	Demonstrated the feasibility of using ctDNA as a surrogate tissue for the presence of *KIT*/*PDGFRA* mutations prior to resection of primary tumor.

Table 2. (Continued)

Author, year	Aim	Patients		Technique	Result
		N **of tumor samples**	***N*** **of plasma samples**		
Boonstra and colleagues[98]	To develop a ddPCR assay to detect common *KIT* exon 11 mutations in both tumor tissue and ctDNA.	Archival FFPE from 27 GISTs	22 (from 22 GIST, taken before start of TKI treatment)	Sanger sequencing (FFPE), NGS (FFPE), ddPCR (FFPE and plasma)	Demonstrated the feasibility of a single ddPCR assay for the detection of multiple *KIT* exon 11 mutations in ctDNA.
Namløs and colleagues[99]	To detect *KIT* and *PDGFRA* mutations with high sensitivity in ctDNA from patients with GISTs through NGS.	Tissue from 50 GIST	44 blood samples from treatment-naïve patients and 6 from GISTs under TKIs	NGS (tissue and plasma)	- Plasma from high-risk patients or with metastatic disease showed more frequently detectable mutations in ctDNA compared with patients with localized or intermediate to low-risk GISTs. - Detection of ctDNA in patients undergoing TKI treatment can be related to the disease development.
Li and colleagues[100]	To investigate feasibility of detecting ANO1 in CTCs in GISTs and association between ANO1 expression and clinical outcome of GIST.		Blood samples from 121 GISTs (of whom, 52 were high-risk GISTs, 42 intermediate risk, 18 low or very low risk), 21 gastric cancer, 23 colorectal cancer patients and 10 healthy controls	qRT-PCR	- ANO1 is a specific marker of CTCs in GISTs -High ANO1 correlated with high risk, large tumor size and high mitotic count - ANO1 positive expression correlated with poor disease-free survival. - In the neoadjuvant setting, reduction of ANO1 expression correlated with the response to imatinib.
Atay and colleagues[101]	To provide a comprehensive proteome analysis and characterization of GIST-derived exosomes that might be used as a resource for the discovery of new diagnostic biomarkers and therapeutic targets.		30 (from 18 GISTs and 12 healthy donors)	Mass spectrometry	Showed proteomic analysis of circulating exosomes is suitable for diagnosis, prognosis and monitoring of treatment response.

BEAMing: bead emulsion amplification and magnetics; cfDNA: circulating free DNA; ctDNA: circulating tumor DNA; ddPCR: digital droplet PCR; FFPE: formalin-fixed paraffin-embedded; GIST, gastrointestinal stromal tumor; PCR, polymerase chain reaction; qRT-PCR: quantitative-real-time reverse transcription PCR; NGS: next-generation sequencing; TKI, tyrosine-kinase inhibitor.

a clonal *KIT* primary mutation is an artefact derived from the NGS methodology. Nevertheless, NGS applications hold the pivotal advantage to detect novel secondary mutations conferring resistance. Therefore, it may represent an enrichment for future studies or clinical trials of novel/repositioned/existing drugs specifically targeting secondary mutations. In the wake of this idea, Wada and coworkers investigated four imatinib-resistant GIST patients, who underwent surgical resection.[96] In particular, the authors analyzed, through an NGS approach, mutations in tumor tissue from resected primary and imatinib-resistant lesions and in ctDNA isolated before and after imatinib treatment. All the four patients had a primary *KIT* exon 11 lesion with deletions involving codons 550 to 559. Patients with imatinib-resistant lesions had resistance mutations in the *KIT* exon 13 ($n = 3$) and exon 18 ($n = 1$); the same genetic alterations were measured in ctDNA.

The non-invasive detection of mutations is pivotal for the selection process of target agents. Indeed, the efficacy of sunitinib correlates with the secondary mutation genotype; specifically, sunitinib is more effective in *KIT* exon 13 or 14-mutant GISTs.[95,102] With regard to the third-line treatment, regorafenib, the GRID study reported the same benefits for patients harboring the most common primary *KIT* mutations. More recently, the study by Ben-Ami and coworkers suggested regorafenib provides long-term benefit in metastatic GIST patients with *KIT* exon 11 primary mutations and WT for *KIT*/*PDGFRA*.[80,83] Overall, it is clearly important to know the tumor secondary mutational status to predict the efficacy of TKIs in imatinib-resistant GISTs. Wada and collaborators evaluated also the cfDNA as a surrogate biomarker of response. Indeed, in the literature, there are different reports evaluating its feasibility in this context.[103] The study by Wada and colleagues, also reported that cfDNA decreased marginally with treatment in two patients, while another patient with stable disease exhibited a substantial increment in the cfDNA concentration. These data pinpoint that the concentration of cfDNA might not accurately reflect tumor evolution.[96] However, we should not under-evaluate that specific tumor markers may be predominantly present in the cell-bound rather than in the cell-free fraction.[104] This issue should be considered with caution as tumor treatment often influences leukocytes or erythrocytes apoptosis, with consequent release of cell-bound DNA into plasma. Therefore, an increase in some markers will be a clue to blood cell death, rather than reflecting tumor growth. Actually, the work by Wada and colleagues in one patient following surgery of primitive lesion, showed that cfDNA increased substantially, while ctDNA was below the threshold of detection before recurrence. After progression of the imatinib-resistant lesion, ctDNA increased and then returned to the value below the threshold following sunitinib treatment, while cfDNA was constantly at high levels. In this regard, ctDNA may be a better biomarker compared with cfDNA.[96] Nonetheless, this has to be taken with caution as ctDNA may reflect a mixed population: it can derive from dying tumor cells responding to therapy or from tumor cells resistant to therapy.[18] Unfortunately, to date, no further reports focusing on the impact of resistance mutations have been published. Taken together, the data led us to speculate on the importance of liquid biopsy to follow the tumor evolution under TKI treatment. However, the available literature is still too scarce, and additional prospective investigations recruiting a major number of patients are critical before its translation into clinical practice in GISTs.

More recently, three papers have focused their attention on the detection of primary mutations in GISTs. In the first of these works, Kang and colleagues[97] analyzed plasma samples, collected before surgery, from 25 patients with localized gastric GISTs. This is one of the few studies addressing the role of ctDNA detection in localized GISTs. The standard treatment for localized GISTs is complete surgical resection; however, mutational status is important for the indication of adjuvant or neoadjuvant imatinib therapy, and occasionally helps in the diagnosis of GISTs. However, tumor tissue samples before surgery can be inadequate for standard mutation analysis. In this context, liquid biopsy may have the potential to detect primary mutations prior to resection. In addition, presence of mutant ctDNA after surgery might allow assessing microscopic residual disease, possibly responsible of recurrence, and guiding adjuvant therapy recommendation. Mutational status of the paired plasma-tissue samples were investigated through Sanger sequencing (tissue) and an NGS panel covering *KIT* exons 9, 11, 13, 17 and *PDGFRA* exon 18 (plasma). A total of 18 out of 25 GISTs were *KIT* exon 11 mutants, and the remaining were *KIT*/*PDGFRA* WT. The reported concordance between plasma and tissue samples was 72%, with 13 patients identified as

KIT exon 11 mutants in plasma. None of the seven *KIT*/*PDGFRA* WT patients had measurable mutations in the plasma DNA.[97] In a subsequent study, Boonstra and collaborators showed digital droplet PCR (ddPCR) may be useful in the detection of common *KIT* exon 11 mutations in both GIST tumor tissue and ctDNA.[98] In particular, the authors used an in-house designed single ddPCR assay covering two hotspots in exon 11. According to COSMIC, around 80% of the mutations in this exon cluster in two hotspot regions of approximately 25 bp within a 100 bp range from each other.[105] The authors first validated ddPCR in 36 pretreatment biopsies of GIST patients previously tested *via* Sanger sequencing or NGS. A total of 27 patients were *KIT* exon 11 mutants, whereas 9 had no *KIT* exon 11 mutations and served as negative controls. ddPCR resulted in 100% of specificity since all controls turned out to be negative, and in 77% of sensitivity, detecting 21/27 mutations. However, five mutations were located within the annealing sequence of the primers; one of the five samples had a duplication that was considered negative even characterized by a typical pattern of droplet distribution. Considering the remaining 22 samples covered by ddPCR, only 1 with a single nucleotide variant located within the detection range of probe 2 was a true false-negative tumor, and therefore the assay showed an overall sensibility of 95% for the regions covered in *KIT* exon 11. Subsequently, the ddPCR assay was tested on plasma samples available before and at multiple time points during imatinib therapy for 14 GIST patients with metastatic disease and 8 with localized GISTs. All the 22 patients had measurable disease before collection of the first (baseline) plasma sample. Analysis of the baseline plasma sample highlighted the presence of a *KIT* exon 11 mutation in 13 of 14 metastasized patients, and only in 1 of 8 with localized disease. The authors also used ddPCR to monitor the treatment response in serial plasma samples from 11 metastasized GISTs under TKI treatment. They showed a decrease in *KIT* exon 11 mutant ctDNA during treatment, which was in agreement with radiological treatment response or stable disease, evaluated according to RECIST criteria.[98]

More lately, Namløs and colleagues applied an NGS approach to analyze ctDNA samples from 44 treatment-naïve GIST patients ($n = 35$ *KIT* and $n = 9$ *PDGFRA* mutants).[99] Somatic mutations in ctDNA were found in 36% of the plasma samples ($n = 16$ patients). The ctDNA detection rate was higher for *KIT* mutants (42.8% mutants detected) compared with *PDGFRA* mutant GISTs (11.1%). In addition, plasma from high-risk patients or with metastatic disease showed more frequently detectable mutations in ctDNA compared with patients with localized or intermediate to low-risk GISTs. Furthermore, the authors showed that ctDNA detection in patients undergoing TKI treatment might be related to disease development. Indeed, analysis on six *KIT* mutant GISTs receiving TKIs at the time of blood collection, revealed the presence of ctDNA in patients with progressive disease; no mutations were observed in patients with stable disease.

CTCs in GIST

Research data on CTCs in GISTs are very scarce, and currently, only one study has been published in the literature.[100] In particular, Li and collaborators investigated the feasibility of detecting ANO1 (known as DOG1) expression in peripheral blood mononuclear cells (PBMCs) of GIST patients. ANO1 is, together with *KIT*, a diagnostic biomarker in GISTs. A total of 54% of the patients analyzed were ANO1-positive and a higher expression was significantly associated with a larger tumor size, high mitotic count and risk. The authors investigated also the prognostic role of ANO1. In particular, ANO1 expression was tested in 112 before and 4 weeks after surgical resection. A total of 51.8% of patients were ANO1-positive pre-resection, and only 12.1% ($n = 7$) of them turned out positive after surgery; these patients were characterized by liver metastasis. Afterwards, ANO1-positive status emerged in 21 GISTs experiencing recurrence after surgery. Finally, the authors evaluated imatinib efficacy after 3 months of neoadjuvant treatment in 26 GIST patients, preoperatively treated with TKIs. ANO1 expression was tested in PBMCs pre and post imatinib treatment. The 17 patients with disease control (partial response or stable disease) showed a reduction trend of ANO1 expression and 10 patients became negative, whereas the expression level did not change in the 9 patients with progressive disease. Despite the limitations of the study, including the sample size, these results showed that CTC detection in PBMCs by quantifying ANO1 could be taken into account and may offer an interesting opportunity to monitor the disease course as well as the clinical response to imatinib.[100]

Circulating vesicles and RNA in GIST

If the literature on circulating DNA in GISTs is quite scarce, reports on the different molecules are anecdotal, representing a new, valid and largely unexplored field of investigation. Up to now, the majority of the studies focused on circulating vesicles. The first evidence of exosome release in GISTs dates back to 2014, when Atay and collaborators investigated the role of exosomes in mediating the complex interplay between the tumor and stroma during disease progression.[101] In particular, they selected the human cell line GIST-T1 as an *in vitro* disease model, expressing the most common type of mutation involved in GIST pathogenesis (i.e. *KIT* exon 11). The authors showed GIST cells secreted high number of exosomes, or 'oncosomes', carrying the activated oncogenic *KIT* receptor. Interestingly, the authors reported that the invasion of stromal cells, through these specific exosomes, led to the production of interstitial cells of Cajal (ICC)-like cells. Indeed, these oncosomes act like phenotypic modifiers of their microenvironment, promoting tumor progression through the regulation of downstream *KIT*-signaling pathways in stromal cells, which differentiate to ICC-like cells. Moreover, conditioning with GIST-T1-derived exosomes promotes enhanced secretion of the matrix metalloproteinase (MMP) 1, which is recognized to dynamically contribute to tumor cell invasion.[101] Even if the authors could not prove the direct contribution of *KIT* in this process due to methodological issues, these preliminary data indicate that tumor transformation in not solely driven by oncogenes but other factors are involved. Indeed, the selective blocking of MMP exosome-mediated, MMP-1 secretion abrogated tumor invasiveness. In other words, this first report highlighted the existence of a feedback loop between a signaling mediated by the exosomes and matrix MMPs and suggests a potential role for exosomes as stroma-modifiers.[101] Subsequently, a work by Junquera and collaborators, described for the first time multivesicular sphere (MVS) production in GIST cells *in vivo*.[106] Specifically, MVSs are spherical membrane structures produced through a budding process from the plasma membrane, containing many MVs referred to as spheresomes. Interestingly, spheresomes are different from exosomes, and represent a novel mechanism coming from a spherical membrane structure. Analyzing eight gastric biopsies from GIST patients, MVSs containing spheresomes were observed establishing interactions with cytoskeleton filaments and the extracellular matrix. In particular, once in the extracellular matrix, medium MVSs can release the spheresomes (remaining empty) or cross the wall of blood vessels near cancer cells, entering the circulation. This last observation contributes to strengthening the evidence that tumor-derived EVs, besides stimulating cells at distant sites in the organism, play a key role in the initiation of the metastatic niche. The idea is that tumor-derived EVs give rise to the receptive microenvironment supporting the cell arrival, engraftment and survival in the metastatic site.[106,107] In addition, Junquera and colleagues observed a considerable variability depending on the tumor sample; in particular, early stage tumors (<4.5 cm and low mitotic activity index) secrete a high number of spheresomes, while tumor with high mitotic activity do not show a presence of spheresomes. This suggests that exacerbation of a specific communication process between mesenchymal cells within tumors could occur, facilitating growth or metastases.[106] Therefore, MVSs may represent a novel and alternative approach to cancer treatment in which MVSs are important therapeutic targets, in a strategy aimed at neutralizing or trapping, thus preventing the signaling process they initiate. After these preliminary studies, the most recent data in GISTs were presented, this year, by Atay and collaborators.[108] In this work, they performed a comprehensive vesicular proteome profiling of GIST-derived exosomes (GDEs), from two GIST cell lines (GIST-T1 and GIST-882), providing important information on the content, biological role and therapeutic value of these vesicles. Specifically, authors showed that GIST cell lines are characterized by an inherent overactive exosome production mechanism, leading to their release and accumulation. Proteomic analysis showed that total exosomal protein content was significantly higher in GDEs compared with the non-transformed primary myometrial smooth muscle cells, representing the host healthy cells surrounding the tumor *in vivo*. In particular, the authors identified a core of 1060 proteins supporting the exosomal origin (e.g. features shared with exosomes-derived from other cells types), while maintaining the tumor identity. Specifically, the core protein was enriched in diagnostic markers and other features related to GISTs as well as novel kinases, phosphatases and tumor-associated antigens, previously unreported in GISTs. Interestingly, among the markers, the authors showed an enrichment of the markers of autophagy, which is involved in GIST survival and progression (for a review see Ravegnini and colleagues[109]). An added value of this work is the evaluation of selected GDE-associated core protein in clinical

specimens, with the capture and isolation, of *KIT* positive (*KIT*$^+$) exosomes from the plasma of GIST patients ($n = 18$) and healthy donors ($n = 12$). In this preliminary analysis, Atay and colleagues showed that the number of *KIT*$^+$ vesicles in controls was small, suggesting the majority of them in GIST patients originate from the tumor.[101] This concept was further supported by the correlation between circulating *KIT*$^+$ levels with tumor burden and treatment response. Indeed, the authors observed that accumulation of circulating *KIT*$^+$ exosomes was: (1) enhanced in the peripheral blood of patients with metastatic GISTs compared with primary disease, and (2) decreased in patients responding to treatment. In view of these considerations, quantitative changes in exosomes might represent a tool to predict malignant capabilities (e.g. recurrence or metastasis) and response to therapy. Overall, the results of this comprehensive proteomic analysis of exosomes secreted by GIST cells have unveiled different clinically relevant candidates to be circulating diagnostic and monitoring-disease biomarkers. Nevertheless, despite an in-depth examination of the proteome of isolated *KIT*$^+$ exosomes holding great promise, the feasibility of the analysis remains to be elucidated.

To date, no additional studies in GISTs described these or additional kinds of circulating molecules. In particular, the literature on circulating miRNAs or lncRNAs is missing, leaving many questions unanswered on their potential role in tumor progression and metastasis, as well as in TKIs response.

A small number of studies on miRNAs and lncRNAs, limited to tissue GISTs (for a review see Nannini and colleagues[110] and Kupcinskas[111]) have showed a few miRNAs and lncRNAs involved in regulating several genes and biological processes in GIST pathogenesis; however, up to now, none of these have been translated to the clinic or obtained from the bloodstream. Nevertheless, in the near future, molecular investigation of miRNA and lncRNA could represent interesting circulating candidates as prognostic, diagnostic and disease monitoring biomarkers.

Liquid biopsy in GISTs: clinical utility and challenging issues

Liquid biopsy has demonstrated to be a valuable tool, and recent technological innovations are generating promising clinical results, suggesting that liquid biopsy might be incorporated into clinical practice in the near future. This is the case of ctDNA analysis in non-small cell lung carcinoma and CTCs analysis in breast and colon cancer.[112–115] However, for other tumors, liquid biopsy is still a wish, highlighting a great lack of homogeneity between the various types of cancer patients. In this context, GISTs, despite the attractive biology based on a few driver mutations, are among the tumors in which the advances in liquid biopsy are very limited. Certainly, even if the studies so far reported do not permit drawing any definitive conclusion, some individual cases underscore that liquid biopsy may be useful in monitoring the clinical response to TKIs in GISTs. The development of resistance mutations is the main mechanism of acquired resistance to TKIs, found in 80–90% of patients experiencing disease progression.[87,116,117] Therefore, the prompt identification of these mutations might be clinically relevant to drive therapeutic decision-making. On the other hand, the application of liquid biopsy to improve GIST early diagnosis appears to be challenging. In particular, the heterogeneity of primary mutations in *KIT* or *PDGFRA* and the relative paucity of circulating elements in the bloodstream clearly impairs the sensitivity. Moreover, the lack of standardized methods of analysis realizes that we are still far behind in the application of liquid biopsy in GISTs. Therefore, in order to broaden the knowledge and application of liquid biopsy in early diagnosis and prognosis, methodological issues need to be addressed. In this regard, *KIT* exon 11 mutations, accounting for approximately 90% of *KIT* mutations, vary remarkably in length and location; consequently, their detection in plasma, through PCR-based methods is quite problematic.[94] Great enthusiasm has emerged regarding NGS applications, and their ability to identify low and ultralow frequency mutations, although this approach involves inherent experimental errors. Indeed, even for methods with the lowest reported error rate, thousands of false positive variants are possible in a fully sequenced human genome.[118] Errors can result from bioinformatics analysis and experimental process (e.g. sample or library preparation and sequencing chemistries). Experimental errors can be reduced through confirmatory sequencing studies, in a manner independent of the algorithms and chemistries used. Nevertheless, NGS applications hold the pivotal advantage of reducing false-negative results, compared with BEAMing and allele-specific L-PCR, which are certainly limited by the identification of

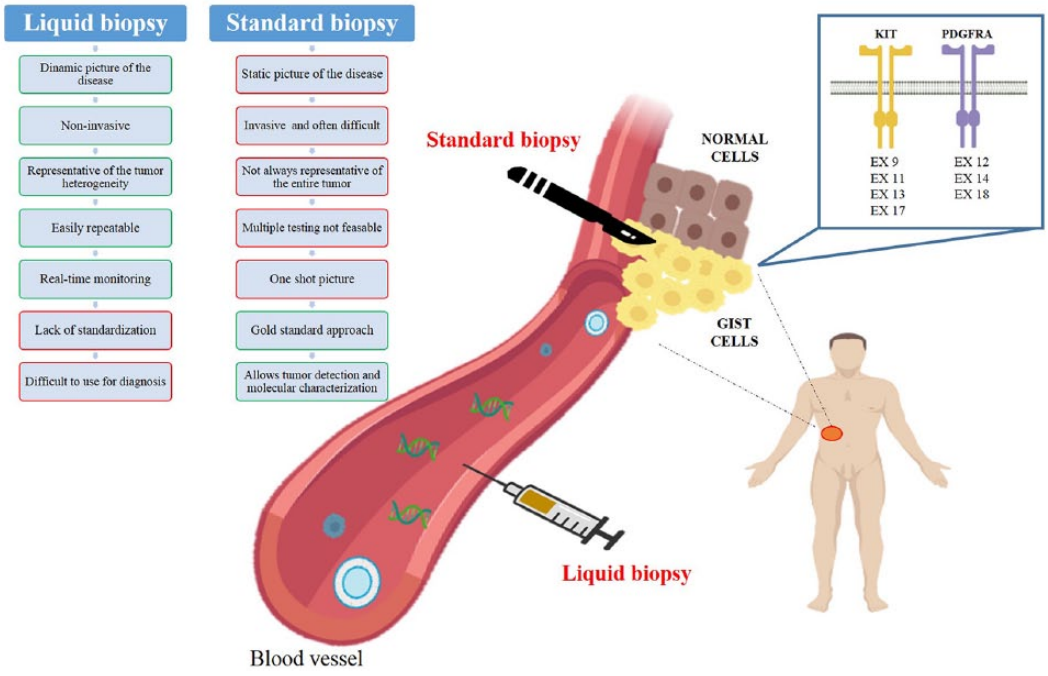

Figure 3. Pros and cons of liquid and standard biopsy in GIST.
GIST, gastrointestinal stromal tumor.

predesigned mutations conferring resistance. In view of this consideration, overcoming this limitation with novel NGS techniques is mandatory. Actually, optimizing target mutation profiling is beneficial for patients, as the identification of known and novel resistance mutations may help in selecting optimal responders for molecular therapies.

Conclusion

The concept of 'oncogene addiction' was first introduced in the late 20th century to describe the constitutive activity of specific activated/overexpressed oncogenes needed for the continuous maintenance of the malignant phenotype.[119] Afterwards, it did not take long to realize that drugs, specifically targeting hyperactivated oncogenes, could selectively kill cancer cells. Overall, this finding paved the way to the era of precision medicine and targeted therapy, which are based on correct patient selection. The study and understanding of biological processes underlying tumor development and progression have deeply changed cancer treatment, as witnessed in GISTs. In the early 2000s, imatinib revolutionized the field of targeted treatment, particularly in a disease in which no effective treatments were available at that time. The identification of a specific gene status (*KIT* and *PDGFRA* mutations) in a precise tumor type (GISTs) enables the selection of patients for targeted therapies. In this panorama, it is extremely important to have tools available for early diagnosis, improving the prognosis, for real-time monitoring of the disease, and ultimately the survival rate.

Currently, tissue biopsy represents the gold standard for a precise diagnosis of cancer; however, this approach suffers from several limitations, summarized in Figure 3. In view of these limits, there is an urgent need of minimally invasive techniques allowing a strict patient follow up at different time points; here originates the concept of liquid biopsy. The development of a 'liquid biomarker', which can be easily isolated from any body fluids, represents a great opportunity for early diagnosis and drug response monitoring. Despite the promising expectation, the research is still in its embryonic phase. Indeed, we can list a series of pros and cons (Figure 3) related to liquid biopsy, whilst for some circulating components, such as exosomes, we are even far from clinical applications.

In conclusion, liquid biopsy has entered the scene of the era of personalized medicine, representing a key tool to complement the other available

techniques routinely used in the clinic. With regard to GISTs, a global effort should be considered as mandatory to translate the use of liquid biopsy into the clinic.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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References

1. Rehm HL. Disease-targeted sequencing: a cornerstone in the clinic. *Nat Rev Genet* 2013; 14: 295–300.
2. Kamps-Hughes N, McUsic A, Kurihara L, *et al*. ERASE-Seq: leveraging replicate measurements to enhance ultralow frequency variant detection in NGS data. *PLoS One* 2018; 13: e0195272.
3. Rabbani B, Nakaoka H, Akhondzadeh S, *et al*. Next generation sequencing: implications in personalized medicine and pharmacogenomics. *Mol Biosyst* 2016; 12: 1818–1830.
4. Alix-Panabieres C and Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov* 2016; 6: 479–491.
5. Bardelli A and Pantel K. Liquid Biopsies, What We Do Not Know (Yet). *Cancer Cell* 2017; 31: 172–179.
6. Siravegna G, Marsoni S, Siena S, *et al*. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol* 2017; 14: 531–48.
7. Bayraktar R, Van Roosbroeck K and Calin GA. Cell-to-cell communication: microRNAs as hormones. *Mol Oncol* 2017; 11: 1673–86.
8. Murtaza M, Dawson S-J, Pogrebniak K, *et al*. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun* 2015; 6: 8760.
9. Crowley E, Di Nicolantonio F, Loupakis F, *et al*. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol* 2013; 10: 472–484.
10. Tseng J-Y, Yang C-Y, Liang S-C, *et al*. Dynamic changes in numbers and properties of circulating tumor cells and their potential applications. *Cancers (Basel)* 2014; 6: 2369–2386.
11. Elazezy M and Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. *Comput Struct Biotechnol J* 2018; 16: 370–378.
12. Reinert T, Schøler L V, Thomsen R, *et al*. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut* 2016; 65: 625–634.
13. Dawson S-J, Tsui DWY, Murtaza M, *et al*. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* 2013; 368: 1199–1209.
14. Murtaza M, Dawson S-J, Tsui DWY, *et al*. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 2013; 497: 108–112.
15. Stewart CM and Tsui DWY. Circulating cell-free DNA for non-invasive cancer management. *Cancer Genet*. 2018; 228–229: 169–179.
16. Stewart CM, Kothari PD, Mouliere F, *et al*. The value of cell-free DNA for molecular pathology. *J Pathol* 2018; 244: 616–627.
17. Heitzer E, Haque IS, Roberts CES, *et al*. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. Epub ahead of print 8 November 2018. DOI: 10.1038/s41576–018–0071–5.
18. Kidess E and Jeffrey SS. Circulating tumor cells versus tumor-derived cell-free DNA: rivals or partners in cancer care in the era of single-cell analysis? *Genome Med* 2013; 5: 70.
19. Wan JCM, Massie C, Garcia-Corbacho J, *et al*. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017; 17: 223–238.
20. Ehrich M, Tynan J, Mazloom A, *et al*. Genome-wide cfDNA screening: clinical laboratory experience with the first 10,000 cases. *Genet Med* 2017; 19: 1332–1337.
21. Mehra N, Dolling D, Sumanasuriya S, *et al*. Plasma cell-free DNA concentration and outcomes from taxane therapy in metastatic castration-resistant prostate cancer from two phase III trials (FIRSTANA and PROSELICA). *Eur Urol* 2018; 74: 283–291.

22. Jahr S, Hentze H, Englisch S, *et al*. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; 61: 1659–1665.

23. Fiala C and Diamandis EP. Utility of circulating tumor DNA in cancer diagnostics with emphasis on early detection. *BMC Med* 2018; 16: 166.

24. Misale S, Yaeger R, Hobor S, *et al*. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012; 486: 532–536.

25. Diaz LA, Williams RT, Wu J, *et al*. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012; 486: 537–540.

26. Jiang ZF, Cristofanilli M, Shao ZM, *et al*. Circulating tumor cells predict progression-free and overall survival in Chinese patients with metastatic breast cancer, HER2-positive or triple-negative (CBCSG004): a multicenter, double-blind, prospective trial†. *Ann Oncol* 2013; 24: 2766–2772.

27. Hiltermann TJN, Pore MM, van den Berg A, *et al*. Circulating tumor cells in small-cell lung cancer: a predictive and prognostic factor. *Ann Oncol Off J Eur Soc Med Oncol* 2012; 23: 2937–2942.

28. Lianidou ES, Markou A and Strati A. The role of CTCs as tumor biomarkers. *Adv Exp Med Biol* 2015; 867: 341–367.

29. Zhang C, Guan Y, Sun Y, *et al*. Tumor heterogeneity and circulating tumor cells. *Cancer Lett* 2016; 374: 216–23.

30. Gwak H, Kim J, Kashefi-Kheyrabadi L, *et al*. Progress in circulating tumor cell research using microfluidic devices. *Micromachines* 2018; 9: 353.

31. Ferreira MM, Ramani VC and Jeffrey SS. Circulating tumor cell technologies. *Mol Oncol* 2016; 10: 374–394.

32. Aceto N, Bardia A, Miyamoto DT, *et al*. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* 2014; 158: 1110–1122.

33. Mitra A, Mishra L and Li S. EMT, CTCs and CSCs in tumor relapse and drug-resistance. *Oncotarget* 2015; 6: 10697–10711.

34. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer* 2007; 7: 834–846.

35. Cabel L, Proudhon C, Gortais H, *et al*. Circulating tumor cells: clinical validity and utility. *Int J Clin Oncol* 2017; 22: 421–430.

36. Anfossi S, Babayan A, Pantel K, *et al*. Clinical utility of circulating non-coding RNAs - an update. *Nat Rev Clin Oncol* 2018; 15: 541–563

37. Cortez MA, Bueso-Ramos C, Ferdin J, *et al*. MicroRNAs in body fluids–the mix of hormones and biomarkers. *Nat Rev Clin Oncol* 2011; 8: 467–477.

38. Arroyo JD, Chevillet JR, Kroh EM, *et al*. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A* 2011; 108: 5003–5008.

39. Vickers KC, Palmisano BT, Shoucri BM, *et al*. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol* 2011; 13: 423–433.

40. Pucci M, Reclusa Asiáin P, Duréndez Sáez E, *et al*. Extracellular vesicles As miRNA nano-shuttles: dual role in tumor progression. *Target Oncol* 2018; 13: 175–187.

41. Kim KM, Abdelmohsen K, Mustapic M, *et al*. RNA in extracellular vesicles. *Wiley Interdiscip Rev RNA* 2017; 8: e1413.

42. Mercer TR, Dinger ME and Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009; 10: 155–159.

43. Ørom UA and Shiekhattar R. Long noncoding RNAs usher in a new era in the biology of enhancers. *Cell* 2013; 154: 1190–1193.

44. Ulitsky I. Interactions between short and long noncoding RNAs. *FEBS Lett* 2018; 592: 2874–2883.

45. Shen X, Qi P and Du X. Long non-coding RNAs in cancer invasion and metastasis. *Mod Pathol* 2015; 28: 4–13.

46. Qi P, Zhou X and Du X. Circulating long non-coding RNAs in cancer: current status and future perspectives. *Mol Cancer* 2016; 15: 39.

47. Grossi E, Sánchez Y and Huarte M. Expanding the p53 regulatory network: LncRNAs take up the challenge. *Biochim Biophys Acta* 2016; 1859: 200–208.

48. Mitchell PS, Parkin RK, Kroh EM, *et al*. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci* 2008; 105: 10513–10518.

49. Cheng G. Circulating miRNAs: Roles in cancer diagnosis, prognosis and therapy. *Adv Drug Deliv Rev* 2015; 81: 75–93.

50. Berindan-Neagoe I, Monroig P, del C, Pasculli B, *et al*. MicroRNAome genome: a treasure for cancer diagnosis and therapy. *CA Cancer J Clin* 2014; 64: 311–336.

51. Redis RS, Calin S, Yang Y, *et al.* Cell-to-cell miRNA transfer: from body homeostasis to therapy. *Pharmacol Ther* 2012; 136: 169–174.
52. Hansen TB, Jensen TI, Clausen BH, *et al.* Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; 495: 384–388.
53. Barrett SP and Salzman J. Circular RNAs: analysis, expression and potential functions. *Development* 2016; 143: 1838–1847.
54. Li Y, Zheng Q, Bao C, *et al.* Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 2015; 25: 981–984.
55. Fanale D, Taverna S, Russo A, *et al.* Circular RNA in exosomes. *Adv Exp Med Biol* 2018; 1087: 109–117.
56. Greene J, Baird A-M, Brady L, *et al.* Circular RNAs: biogenesis, function and role in human diseases. *Front Mol Biosci* 2017; 4: 38.
57. Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest* 2016; 126: 1208–1215.
58. Lee I, Baxter D, Lee MY, *et al.* The importance of standardization on analyzing circulating RNA. *Mol Diagn Ther* 2017; 21: 259–268.
59. Lane RE, Korbie D, Hill MM, *et al.* Extracellular vesicles as circulating cancer biomarkers: opportunities and challenges. *Clin Transl Med* 2018; 7: 14.
60. Becker A, Thakur BK, Weiss JM, *et al.* Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell* 2016; 30: 836–848.
61. Kastelowitz N and Yin H. Exosomes and microvesicles: identification and targeting by particle size and lipid chemical probes. *Chembiochem* 2014; 15: 923–928.
62. Zhang W, Xia W, Lv Z, *et al.* Liquid biopsy for cancer: circulating tumor cells, circulating free DNA or exosomes? *Cell Physiol Biochem* 2017; 41: 755–768.
63. György B, Szabó TG, Pásztói M, *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011; 68: 2667–2688.
64. Chen Y, Li G and Liu M-L. Microvesicles as emerging biomarkers and therapeutic targets in cardiometabolic diseases. *Genomics Proteomics Bioinformatics* 2018; 16: 50–62.
65. Théry C, Ostrowski M and Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009; 9: 581–593.
66. Latifkar A, Cerione RA and Antonyak MA. Probing the mechanisms of extracellular vesicle biogenesis and function in cancer. *Biochem Soc Trans* 2018; 46: 1137–1146.
67. Raposo G and Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013; 200: 373–383.
68. De Toro J, Herschlik L, Waldner C, *et al.* Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. *Front Immunol* 2015; 6: 203.
69. Pantel K and Alix-Panabieres C. Real-time liquid biopsy in cancer patients: fact or fiction? *Cancer Res* 2013; 73: 6384–6388.
70. Corless CL, Barnett CM and Heinrich MC. Gastrointestinal stromal tumours: origin and molecular oncology. *Nat Rev Cancer* 2011; 11: 865–878.
71. Maleddu A, Pantaleo MA, Nannini M, *et al.* The role of mutational analysis of KIT and PDGFRA in gastrointestinal stromal tumors in a clinical setting. *J Transl Med* 2011; 9: 75.
72. Pantaleo MA, Astolfi A, Di Battista M, *et al.* Insulin-like growth factor 1 receptor expression in wild-type GISTs: a potential novel therapeutic target. *Int J cancer* 2009; 125: 2991–2994.
73. Corless CL, Fletcher JA and Heinrich MC. Biology of gastrointestinal stromal tumors. *J Clin Oncol* 2004; 22: 3813–3825.
74. Pantaleo MA, Urbini M, Indio V, *et al.* Genome-wide analysis identifies MEN1 and MAX mutations and a neuroendocrine-like molecular heterogeneity in quadruple WT GIST. *Mol Cancer Res* 2017; 15: 553–562.
75. Pantaleo MA, Ravegnini G, Astolfi A, *et al.* Integrating miRNA and gene expression profiling analysis revealed regulatory networks in gastrointestinal stromal tumors. *Epigenomics* 2016; 8: 1347–1366.
76. Angelini S, Ravegnini G, Fletcher JA, *et al.* Clinical relevance of pharmacogenetics in gastrointestinal stromal tumor treatment in the era of personalized therapy. *Pharmacogenomics* 2013; 14: 941–956.
77. Demetri GD, von Mehren M, Blanke CD, *et al.* Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; 347: 472–480.
78. Serrano C, Wang Y, Mariño-Enríquez A, *et al.* KRAS and KIT gatekeeper mutations confer polyclonal primary imatinib resistance in GI stromal tumors: relevance of concomitant

phosphatidylinositol 3-kinase/AKT dysregulation. *J Clin Oncol* 2015; 33: e93–e96.

79. Ravegnini G, Urbini M, Simeon V, *et al.* An exploratory study by DMET array identifies a germline signature associated with imatinib response in gastrointestinal stromal tumor. *Pharmacogenomics J*. Epub ahead of print 20 September 2018. DOI: 10.1038/s41397–018–0050–4.

80. Angelini S, Ravegnini G, Nannini M, *et al.* Folate-related polymorphisms in gastrointestinal stromal tumours: susceptibility and correlation with tumour characteristics and clinical outcome. *Eur J Hum Genet* 2015; 23: 817–823.

81. Demetri GD, van Oosterom AT, Garrett CR, *et al.* Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet (London, England)* 2006; 368: 1329–1338.

82. Ravegnini G, Nannini M, Zenesini C, *et al.* An exploratory association of polymorphisms in angiogenesis-related genes with susceptibility, clinical response and toxicity in gastrointestinal stromal tumors receiving sunitinib after imatinib failure. *Angiogenesis* 2017; 20:139–148.

83. George S, Wang Q, Heinrich MC, *et al.* Efficacy and safety of regorafenib in patients with metastatic and/or unresectable GI stromal tumor after failure of imatinib and sunitinib: a multicenter phase II trial. *J Clin Oncol* 2012; 30: 2401–2407.

84. Ravegnini G, Sammarini G, Angelini S, *et al.* Pharmacogenetics of tyrosine kinase inhibitors in gastrointestinal stromal tumor and chronic myeloid leukemia. *Expert Opin Drug Metab Toxicol* 2016; 12: 733–742.

85. Ravegnini G, Nannini M, Sammarini G, *et al.* Personalized medicine in Gastrointestinal Stromal Tumor (GIST): clinical implications of the somatic and germline DNA analysis. *Int J Mol Sci* 2015; 16: 15592–15608.

86. Serrano C, George S, Valverde C, *et al.* Novel insights into the treatment of imatinib-resistant gastrointestinal stromal tumors. *Target Oncol* 2017; 12: 277–288.

87. Liegl B, Kepten I, Le C, *et al.* Heterogeneity of kinase inhibitor resistance mechanisms in GIST. *J Pathol* 2008; 216: 64–74.

88. Wardelmann E, Merkelbach-Bruse S, Pauls K, *et al.* Polyclonal evolution of multiple secondary kit mutations in gastrointestinal stromal tumors under treatment with imatinib mesylate. *Clin Cancer Res* 2006; 12: 1743–1749.

89. Castellanos-Rizaldos E, Grimm DG, Tadigotla V, *et al.* Exosome-based detection of EGFR T790M in plasma from non-small cell lung cancer patients. *Clin Cancer Res* 2018; 24: 2944–2950.

90. Demetri GD, Jeffers M, Reichardt P, *et al.* Mutational analysis of plasma DNA from patients (pts) in the phase III GRID study of regorafenib (REG) versus placebo (PL) in tyrosine kinase inhibitor (TKI)-refractory GIST: correlating genotype with clinical outcomes. *J Clin Oncol* 2013; 31: (15 Suppl.) ASCO abstracts 10503.

91. www.clinicaltrial.gov.

92. Maier J, Lange T, Kerle I, *et al.* Detection of mutant free circulating tumor DNA in the plasma of patients with gastrointestinal stromal tumor harboring activating mutations of CKIT or PDGFRA. *Clin Cancer Res* 2013; 19: 4854–4867.

93. Yoo C, Ryu M-H, Na YS, *et al.* Analysis of serum protein biomarkers, circulating tumor DNA, and dovitinib activity in patients with tyrosine kinase inhibitor-refractory gastrointestinal stromal tumors. *Ann Oncol Off J Eur Soc Med Oncol* 2014; 25: 2272–2277.

94. Bauer S, Herold T, Muhlenberg T, *et al.* Plasma sequencing to detect a multitude of secondary KIT resistance mutations in metastatic gastrointestinal stromal tumors (GIST). In: *Annual Meeting of the American-Society-of-Clinical-Oncology (ASCO)*, 2015.

95. Kang G, Bae BN, Sohn BS, *et al.* Detection of KIT and PDGFRA mutations in the plasma of patients with gastrointestinal stromal tumor. *Target Oncol* 2015; 10: 597–601.

96. Wada N, Kurokawa Y, Takahashi T, *et al.* Detecting secondary C-KIT mutations in the peripheral blood of patients with imatinib-resistant gastrointestinal stromal tumor. *Oncology* 2016; 90: 112–117.

97. Kang G, Sohn BS, Pyo J-S, *et al.* Detecting primary KIT mutations in presurgical plasma of patients with gastrointestinal stromal tumor. *Mol Diagn Ther* 2016; 20: 347–351.

98. Boonstra PA, ter Elst A, Tibbesma M, *et al.* A single digital droplet PCR assay to detect multiple KIT exon 11 mutations in tumor and plasma from patients with gastrointestinal stromal tumors. *Oncotarget* 2018; 9: 13870–13883.

99. Namløs HM, Boye K, Mishkin SJ, *et al.* Noninvasive detection of ctDNA reveals intratumor heterogeneity and is associated with tumor burden in gastrointestinal stromal tumor. *Mol Cancer Ther* 2018; 17: 2473–2480.

100. Li Q, Zhi X, Zhou J, *et al.* Circulating tumor cells as a prognostic and predictive marker in gastrointestinal stromal tumors: a prospective study. *Oncotarget* 2016; 7: 36645–36654.

101. Atay S, Banskota S, Crow J, *et al.* Oncogenic KIT-containing exosomes increase gastrointestinal stromal tumor cell invasion. *Proc Natl Acad Sci* 2014; 111: 711–716.

102. Heinrich MC, Owzar K, Corless CL, *et al.* Correlation of kinase genotype and clinical outcome in the North American Intergroup Phase III Trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 Study by Cancer and Leukemia Group B and Southwest Oncology Gr. *J Clin Oncol* 2008; 26: 5360–5367.

103. Wimberger P, Roth C, Pantel K, *et al.* Impact of platinum-based chemotherapy on circulating nucleic acid levels, protease activities in blood and disseminated tumor cells in bone marrow of ovarian cancer patients. *Int J cancer* 2011; 128: 2572–2580.

104. Skvortsova TE, Rykova EY, Tamkovich SN, *et al.* Cell-free and cell-bound circulating DNA in breast tumours: DNA quantification and analysis of tumour-related gene methylation. *Br J Cancer* 2006; 94: 1492–1495.

105. Forbes SA, Beare D, Gunasekaran P, *et al.* COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015; 43: D805–D811.

106. Junquera C, Castiella T, Muñoz G, *et al.* Biogenesis of a new type of extracellular vesicles in gastrointestinal stromal tumors: ultrastructural profiles of spheresomes. *Histochem Cell Biol* 2016; 146: 557–567.

107. Graves LE, Ariztia E V, Navari JR, *et al.* Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer Res* 2004; 64: 7045–7049.

108. Atay S, Wilkey DW, Milhem M, *et al.* Insights into the proteome of gastrointestinal stromal tumors-derived exosomes reveals new potential diagnostic biomarkers. *Mol Cell Proteomics* 2018; 17: 495–415.

109. Ravegnini G, Sammarini G, Nannini M, *et al.* Gastrointestinal stromal tumors (GIST): facing cell death between autophagy and apoptosis. *Autophagy* 2017; 13: 452–463.

110. Nannini M, Ravegnini G, Angelini S, *et al.* MiRNA profiling in gastrointestinal stromal tumors: implication as diagnostic and prognostic markers. *Epigenomics*; 72015; 7: 1033–1049.

111. Kupcinskas J. Small molecules in rare tumors: emerging role of MicroRNAs in GIST. *Int J Mol Sci* 2018; 19: 397.

112. Hardingham JE, Grover P, Winter M, *et al.* Detection and clinical significance of circulating tumor cells in colorectal cancer–20 years of progress. *Mol Med* 2015; 21(Suppl. 1): S25–S31.

113. Ignatiadis M, Rack B, Rothé F, *et al.* Liquid biopsy-based clinical research in early breast cancer: the EORTC 90091–10093 treat CTC trial. *Eur J Cancer* 2016; 63: 97–104.

114. Manicone M, Poggiana C, Facchinetti A, *et al.* Critical issues in the clinical application of liquid biopsy in non-small cell lung cancer. *J Thorac Dis* 2017; 9: S1346–S1358.

115. Lim SY, Lee JH, Diefenbach RJ, *et al.* Liquid biomarkers in melanoma: detection and discovery. *Mol Cancer* 2018; 17: 8.

116. Maleddu A, Pantaleo MA, Nannini M, *et al.* Mechanisms of secondary resistance to tyrosine kinase inhibitors in gastrointestinal stromal tumours (Review). *Oncol Rep* 2009; 21: 1359–1366.

117. Gounder MM and Maki RG. Molecular basis for primary and secondary tyrosine kinase inhibitor resistance in gastrointestinal stromal tumor. *Cancer Chemother Pharmacol* 2011; 67: 25–43.

118. Robasky K, Lewis NE and Church GM. The role of replicates for error mitigation in next-generation sequencing. *Nat Rev Genet* 2014; 15: 56–62.

119. Weinstein IB. Cancer. Addiction to oncogenes–the Achilles heal of cancer. *Science* 2002; 297: 63–64.