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Liquid biopsy in colorectal carcinoma:
the search for potential prognostic and
predictive biomarkers

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A bstract

Background: Liquid biopsy is considered a repeatable, non-invasive and dynamic tool. It could be able to recover from liquid samples (expecially blood)) cancer-specific informations (miRNAs, circulating-free DNA (cfDNA), circulating tumor DNA (ctDNA) and exosomes) by overcoming the limitations associated with traditional tissue biopsy.

Aim: to investigate the potential prognostic and predictive role of blood cancer-related biomarkers such as cfDNA or ctDNA and exosomal DNA in colorectal cancer (CRC) patients.

Results: cfDNA or ctDNA and exosomal DNA could have a potential applicability in CRC management. New generation technologies are able to identify clinically relevant genomic alterations with performance comparable to tissue standards.

S

ummary

Malignant neoplasms are one of the world major causes of death and in 2018 it rose from third to second place after cardiovascular disease in USA. According to the AIRTUM data in Italy, colorectal cancer (CRC) is in second place for incidence and mortality both among men (16% of all new tumors and 11% of mortality cancer-related) and among women (13% and 12 % respectively). The genetic-molecular analysis of CRC has led to the identification of different genes and pathways involved in the process of tumor transformation. Mutations in some of these genes have an important prognostic and predictive role, such as to be introduced into clinical practice. Numerous reports have recently shown how the liquid biopsy used in oncology field can be a potential new alternative to traditional tissue biopsy, thanks to its tumor-derived informations as circulating tumor DNA (ctDNA), circulating-free DNA (cfDNA), exosomes and microRNAs. The advantages that have been reported, for liquid biopsy, are mainly related to its easy repeatability and the possibility of avoiding the selection bias related to tumor heterogeneity. Moreover, gene study requires neoplastic tissue for further genetic analysis, but in some conditions, the impossibility of a re-biopsy (because it is technically not feasible or because the patient is deemed unfit for invasive procedure) could lead to situations of “undertreatment” that could be avoided with the introduction of liquid biopsy in clinical practice. This new tool could potentially be able to improve CRC management during diagnosis, real-time monitoring or acquired resistance phases.

In this manuscript it has been described some clinical potential application of ctDNA or exosomal DNA in CRC. Forty CRC patients were enrolled during treatment with chemotherapy with or without the addition to a targeted agent (anti-VEGF or anti-EGFR) depending on the mutational status of RAS assessed on tumor tissue. Blood samples have been collected at the time of enrollment and at subsequent timepoints corresponding to the

radiological reevaluations assessed with computed tomography and tumor markers. The results show that patients with a concentration of cfDNA [cfDNA] greater than the median value (0.47 ng / μ l) had a poor prognosis. Furthermore, it was shown that higher [cfDNA] were associated with mucinous histotype (with unfavorable prognosis) and that there were no differences in [cfDNA] on the basis of the primary CRC sidedness (right versus left). Subsequent analyses showed that [cfDNA] was higher in patients who had progressive disease, although any difference was underlined between those who had been subjected to anti-EGFR rather than anti-VEGF agents. A NGS analysis was performed on the blood of 9 patients. The results suggested that tumor mutational load could correlate with progression-free survival (PFS) and with disease-specific survival (DSS). Subsequently, the exosomal DNA of a patient progressing during adjuvant therapy was studied. From the comparison with cfDNA it would seem that the exosomes should be object of more in-depth studies as they could be able to highlight RAS mutations earlier than cfDNA. The performance of liquid biopsy is clearly dependent on the performance of the technology used to study its components. Therefore, a systematic review of the data with meta-analysis in order to evaluate the diagnostic accuracy of ctDNA in detecting RAS mutations (KRAS or KRAS / NRAS) was performed. The results indicate a good overall performance of liquid biopsy (in terms of AUC, sensitivity and specificity) and the sensitivity analysis did not show bias related to the heterogeneity of the technologies under investigation (PCR, BEAMing, NGS). Furthermore, this pooled analysis suggests a potential different role of liquid biopsy depending on whether the mutational status is unknown (NGS) rather than already known for its real-time monitoring (BEAMing, ddPCR). The meta-analysis therefore provides for the first time a clear and comprehensive evidence of feasibility and good performance of ctDNA RAS testing in mCRC patients providing a strong rationale for its development into randomized clinical trials to validate liquid biopsy in CRC current management.

CHAPTER 1

BACKGROUND

Extracted from:

“Liquid biopsy in cancer patients: the hand lens to investigate tumor evolution”

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Malignant neoplasms are one of the world major causes of death and in 2018 it rose from third to second place after cardiovascular disease in USA. Despite recent advances in prevention and treatment options for many types of cancer, increased life expectancy and the spread of risk factors such as smoking, obesity and eating habits are leading to an increasing incidence and prevalence of malignant neoplasms [1]. In 2025 about 20 million new cases are expected, of which the greatest contribution will come from developing countries, mainly due to the process of "Westernization" of the latter's lifestyle[2] (Figure 1). Colorectal cancer (CRC) is one of the most frequent malignant neoplasms in the world, with greater mortality in developing countries [2]. According to the AIRTUM data in Italy, CRC is in second place for incidence and mortality both among men (16% of all new tumors and 11% of mortality cancer-related) and among women (13% and 12 % respectively). A significant variability emerges from the evaluation and comparison of CRC incidence and mortality rates in different countries. Incidence rates have increased in

Latin American, Asian and Eastern European countries, where these were historically low, due to the rapid spread in these countries of risk factors typical of developed countries[3]. On the other hand, there has been a reduction in the trends of incidence and mortality in Western countries such as the USA thanks to the diffusion of prevention and screening programs[4]. The colorectal carcinogenic evolution is marked by the succession of well-known phases characterized by the progressive accumulation of genetic and epigenetic mutations in addition to histological transformation of the normal colonic mucosa into adenocarcinoma. Two different sequences have been identified (Figure 2). One is the "adenoma-carcinoma sequence" described by Fearon and Vogelstein, which predicts the evolution of the normal intestinal glandular epithelium in tubular adenoma and finally in adenocarcinoma. The other, more recently described, forms a sessile serrated adenoma as pre-neoplastic lesion. Several studies have shown how these two pathways differ for genetic alterations other than histological patterns[5]. Three different pathways of genomic and epigenomic instability have been identified and studied: chromosomal instability, microsatellite instability and epigenetic instability[6]. The most common form of genomic instability is chromosomal instability (Chromosomal Instability, CIN), due to the presence of severe chromosomal abnormalities that lead to aneuploidy.

In the carcinomas associated with CIN the alteration of the APC / β catenin / Wnt pathway is frequent; which represents an early event in the "adenoma-carcinoma sequence", is found in 70% of tubular adenomas, in addition to being responsible for the hereditary FAP syndrome. The APC gene is an oncosuppressor, which encodes a protein that negatively regulates the Wnt pathway, which promotes the degradation of the β catenin by the proteasome system. Functional deactivation of APC leads to uncontrolled activation of the Wnt pathway, resulting in increased transcription of the β catenin target genes, which promote cell proliferation. The RAS protooncogene encodes a family of small proteins, including KRAS and NRAS, capable of inducing GDP-GTP exchange via ATP. These proteins are inserted, along with others, in the EGFR-mediated signal transduction pathway, which controls cell survival and proliferation processes and is often overactive in colorectal carcinoma. KRAS is the most frequently mutated gene in human carcinomas and in about 40% of colorectal cancers. Mutations fall mainly in exon, codon 12 and 13, while more rarely mutations affect exons 3, 4 and the NRAS gene.

The KRAS gene mutation occurs after that of the APC gene within the "adenoma-carcinoma sequence" and still represents an early event in the tumorigenic sequence. The loss of function of the ATPase activity, intrinsic to KRAS, causes the accumulation of proteins in the active conformation bound to the GTP, leading to the constitutive activation of RAF, MAPK, independently from the binding of the EGF to its receptor. As will be explained later, this explains both the loss of efficacy of the use of anti-EGFR monoclonal antibodies and the predictive role of response to therapy given by the knowledge of the mutation of the KRAS gene. The alterations along the PI3K / AKT / mTOR path intervene in the transition from adenoma to carcinoma. In 32% of cases the mutation affects the PIK3CA catalytic subunit, while more rarely it concerns the tumor suppressor PTEN, which negatively regulates the activity of PI3K.

Because this signal transduction pathway is modulated by the EGFR signal, by means of KRAS activation, it is plausible to think of a role, as a predictor of response to anti-EGFR, also by PI3K and PTEN[7]. The TP53 gene is a tumor suppressor that plays a crucial role in maintaining genomic stability through control of the cell cycle G1 / S checkpoint. The mutations of this gene, with loss of function of its protein product (p53), occur in many neoplasms, more typically in the advanced stages of the carcinogenic process. In fact, in colo-rectal carcinoma TP53 is changed only in 5% of adenomas while instead up to 75% of advanced carcinomas[8]. Microsatellite instability (MSI) is found in 15% of sporadic cases of colo-rectal carcinoma and is the main genetic alteration of Lynch syndrome. MSI is due to the inactivation of the DNA Mismatch Repair (MMR) genes such as MLH-1 and MSH-2, leading to the development of a hypermutable phenotype characterized by genomic instability and progressive accumulation of mutations. Tumor tissue mutational profile in association with some clinical parameters define patient prognosis as well as predict the efficacy of new generation targeted monoclonal antibodies (moAbs) such as Anti-Vascular Endothelial Growth Factor (anti-VEGF) or Anti-Epidermal Growth Factor Receptor when used in association with the classic backbone protocols, showing significant benefit on CRC patients survival. Among these:

- 1) The staging, on the basis of the TNM classification (I→IV). However, even within the same TNM stage of disease, there is a significant variability in the outcome of the patient, for which other prognostic factors have been identified to allow a better prognostic stratification of the patient in such a way as to start it to the most appropriate therapies[9]. In particular, the detection of the number of metastatic lymph nodes on the operative specimen represents a fundamental prognostic factor in CRC and remains one of the main criteria on which to base the choice of adjuvant therapy in localized disease[10].
- 2) Tumor grading, that is a prognostic factor independent of the TNM stage. It is based on the cyto-histological evaluation of the glandular contents of the neoplasm and allows to distinguish: well-differentiated tumors (G1); moderately differentiated (G2); poorly differentiated (G3) and undifferentiated (G4) to the progressive decrease of this content[9].
- 3) The involvement of the visceral peritoneum (T4a) and the parietal peritoneum (M1c) is an important independent negative prognostic factor, also able to loco-regional relapse[11].
- 4) As reported previously, the mucinous and signet-ring types have a poor prognosis[12]. Furthermore, the mucinous histological type is predictive of poor response to chemotherapy and biological drugs.
- 5) Other pathological features such as: per neural infiltration, lymph vascular invasion, tumor regression grade (TRG) in carcinomas in the rectum and tumor budding are associated with a worse prognosis[9].
- 6) Carcinoembryonic antigen or CEA is today considered the best tumor marker for stages I-III of CRC undergoing to an adjuvant treatment. A recent study highlighted the usefulness of preoperative CEA measurement in this patient setting. Of the 137.381 patients included, 34% had a high CEA value before surgery and the remaining 66% CEA within normal limits. Their median survival was 70 and 100 months respectively, confirming the negative prognostic value of a high level of preoperative CEA[13]. The CEA

is also used in post-operative monitoring. Its blood concentration normally returns to normal after 4/6 weeks after surgery and its increase could represent a biomarker of early recurrence after tumor resection[14]. CA 19.9 is also used as a negative prognostic marker and is more related to distant metastases[15].

- 7) The clinical condition of the patient at the time of diagnosis and during the illness naturally affects the prognostic point of view. Performance status (PS), measured through suitable items, is among the most used indices for the assessment of the patient's physical well-being.
- 8) Some blood parameters such as: LDH; serum albumin; WBC; hemoglobin; platelets; alkaline transaminase and phosphatase appear to be correlated with prognosis. However, to date none of these parameters is used as a prognostic marker[15].

The genetic-molecular analysis of CRC has led to the identification of different genes and pathways involved in the process of tumor transformation. Mutations in some of these genes have an important prognostic and predictive role, such as to be introduced into clinical practice[16]. Chromosomal instability (CIN) is associated with an unfavorable prognosis, in particular a partial deletion of the long arm of chromosome 18, in which genes encoding major oncosuppressors of the TGF- β pathway such as SMAD2, SMAD4 and SMAD7 are present. been associated with a poor response to 5-FU.

CRC with microsatellite instability (MSI) show a significantly better prognosis than non-MSI and CIN carcinomas. However, MSI tumors are responsible for resistance to 5-FU-based treatments, but they enjoy a good response to other therapeutic regimens.

EGFR signal transduction pathway genes were among the most studied markers, especially following the introduction of targeted therapy based on anti-EGFR monoclonal antibodies. Anti-EGFR acts by binding to the receptor by internalizing it, thus extinguishing this proliferation and cell survival promoter pathway. A mutation affecting one of the genes involved in this pathway, including that of KRAS, is the most frequent, leading to the activation of this signaling pathway independently of EGFR, thus determining the failure of the anti-EGFR. KRAS and NRAS mutations are predictive of failure to respond to anti-EGFR therapy (Figure 3). The same mechanism is considered valid for the BRAF and PIK3CA mutation and for the loss of PTEN function. Furthermore, KRAS and BRAF mutations are associated with a worse prognosis. The dosing and sequencing of the circulating tumor DNA, as we will see later, is among the most promising biomarkers, endowed with negative prognostic value, within the liquid biopsy field[17].

1.2 Liquid Biopsy

In the last years, the comprehension on the tumor molecular biology has allowed to understand the mechanisms underlying the tumor carcinogenesis, identifying some genes (e.g., RAS, BRAF, PI3K) whose mutation status is associated with a different prognosis[18]. The same has been shown in case of high degree of microsatellite instability (MSI-H) detection. The mixture of all these new parameters within

different classifications allowed to hypothesize four molecular subtypes of CRC with different characteristics and biological behavior: CMS1 (characterized by microsatellite instability 15%), CMS2 (standard and characterized by the overexpression of WNT and MYC signal-dependent pathways, 35%), CMS3 (with metabolic dysregulation, 15%), and finally CMS4 (mesenchymal type, with overexpression of factors derived from mesenchyme-regulating angiogenesis and stromal invasion, 25%). The remaining portion (about 10%) can be defined by mixed characteristic. Therefore, liquid biopsy, studying features of circulating tumor cells (CTCs), circulating-free DNA (cfDNA), tumor DNA (ctDNA) exosomes, and microRNAs (miRNAs), could represent in the next future an interesting tool useful to help oncologists in the management of CRC patients (Figure 4).

1.3 Circulating-free DNA (cfDNA) and Circulating Tumor DNA (ctDNA)

Numerous reports have recently shown how the liquid biopsy used in oncology field could be a potential new alternative to traditional tissue biopsy, thanks to tumor-derived genetic material as circulating tumor DNA (ctDNA), circulating-free DNA (cfDNA), exosomes and microRNAs[19]. The advantages that have been reported, for liquid biopsy, are mainly related to its easy repeatability and the possibility of avoiding the selection bias related to tumor heterogeneity (Figure 5). CfDNA is one of the liquid biopsy component. A portion of cfDNA that can sometimes reach 10% (0.01% to 90%) of the total is represented by the ctDNA, the amount of which also varies according to the type of neoplasm as well as for inter-patient heterogeneity. This genetic material is released into the bloodstream by means of passive (especially apoptosis, necrosis or macrophage phagocytosis) or active (still little known) mechanisms. The nucleosome spacing (150-180 bp) is very rich in cfDNA which makes it a potential neoplastic biomarker. The quantization and study of the properties of the cfDNA have associated its concentration with some important clinical parameters such as the stage, vascularization and disease burden as well as the response to antitumor chemotherapy[20, 21]. In addition, cfDNA has been shown to be able to identify clinically relevant somatic genomic mutations such as KRAS, NRAS, BRAF, EGFR. This type of test is the easy to search for driver mutations since these are tumor specific[22]. cfDNA can be detected either in healthy subjects (e.g., suffering from inflammatory diseases or infections) or from cancer patients. Nevertheless, in the latter case, its concentration may be related to biological characteristics such as tumor size and tumor growth rate, although several authors have assumed a role in the pathogenesis of distant metastases. There are numerous techniques to identify the cfDNA. Among these, more recently, the most used are represented by the quantitative real-time PCR (qPCR), digital polymerase chain reaction (dPCR), BEAMing and the so-called next generation sequencing (NGS) techniques. A possible limitation is due to a high rate of heterogeneity that makes difficult to compare the results of different clinical trials. The importance of cfDNA, especially of ctDNA component, has been demonstrated in a number of reports, which also took into consideration colorectal tumors in several clinically relevant aspects[23, 24]. One of

these is the monitoring of the minimal residual disease. As mentioned in the previous paragraph, the CRC risk of recurrence after primary surgery remains still significant especially within the first 5 years for stages II and III of Dukes at around 30% and 50%, respectively, probably caused by the presence of micrometastatic spread. It would seem that detection of ctDNA may constitute a valid instrument able to help clinicians to identify groups of patients as a high risk of recurrence to design an appropriate adjuvant strategy, especially regarding the management of stage II that is the most controversial in CRC. In this light, the droplet digital PCR (ddPCR) would appear to be an effective technique with high sensitivity and specificity in predicting the risk of recurrence after radical surgery or after adjuvant strategy[25]. In a study by Tie J et al., it is reported that in 78 patients with stage II diagnosed CRC, the persistence of ctDNA in radically resected patients is associated with disease recurrence, suggesting ctDNA as a possible valid biomarker of tumor recurrence[26]. The addition of targeted agents to conventional chemotherapy has been an important breaking point in the treatment of solid tumors, including CRC, in which they have considerably increased mOS in several large cohort studies. Panitumumab and cetuximab are two monoclonal antibodies that are very effective in the treatment of wild type mCRC (both K- and N-RAS genes). The first experiences in this setting had shown that the detection of a mutation of exon 2 of the KRAS (codons 12,13) present in approximately 40–45% of cases conferred resistance to treatment with this class of drugs. Subsequent retrospective evaluations have also concluded that other mutations in KRAS and other genes from the same family were responsible for a further 17% of resistance to these moAbs molecules (KRAS exons 3,4; NRAS exons 2,3,4) and that probably also some BRAF mutations. Therefore, it has become mandatory to evaluate the mutational status of these genes in order to predict the clinical efficacy and the primary resistance. Routinely, this evaluation is performed on the tissue sample. A possible role of the cfDNA might be to predict in advance and in a less invasive way the response to these targeted agents and the emergence of any resistance, before imaging techniques, thus avoiding unnecessary and potentially toxic treatments to patients. Innovations in the field of molecular biology are leading to a continuous evolution of treatment strategies for CRC treatment, and this phenomenon is largely attributable to the potentially “driver” role of new gene on which next-generation drugs are targeted. The gene study requires neoplastic tissue for further genetic analysis, but in some conditions, the impossibility of a re-biopsy (because it is technically not feasible or because the patient is deemed unfit for invasive procedure) could lead to situations of “undertreatment” that could be avoided with the introduction of liquid biopsy in clinical practice. In particular, Thierry et al. demonstrated that the concordance of KRAS and BRAF mutational status between tissue and plasma samples, using qPCR-based technologies, was around 96% [27]. The previous results agree with what was reported by Kidess et al. regarding the identification of the tumor and plasma ctDNA using the SCODA (sequence specific synchronous coefficient of drag alteration) assay [28]. Several authors have speculated on how ctDNA can also be used for assessing treatment response in combination with conventional radiology techniques, demonstrating that a decrease in tumor burden corresponds to a great decrease of ctDNA, even in the early

cycles of chemotherapy[29]. The same role has been suggested by Spindler et al., which reported that an increase in ctDNA levels during treatment with cetuximab was able to anticipate the radiological disease progression[30]. There are, however, limitations to the use of the ctDNA in this field since increased levels could also be a consequence of benign disease (such as inflammation). Further investigations have tested the use of ctDNA in predicting the emergence of resistance during anti-EGFR treatment. It has been observed that there are different molecular mechanisms responsible for primary or secondary resistance to cetuximab or panitumumab in mCRC that take into account both KRAS mutations as previously reported and the involvement of other genes, such as PTEN, able to bypass the signal from EGFR and to activate the PI3K/AKT/mTOR pathway and other mechanisms that include the amplification of HER2, the activation of IGF-1R, and MET amplification. Undoubtedly, however, the main secondary resistance mechanism is dependent on the RAS mutations that arise during treatment with anti-EGFR after selective pressure (about 40% of cases). Identifying the occurrence of these conditions through the use of ctDNA has led several authors to speculate that ctDNA can somehow anticipate the appearance of radiographic progression[31-33]. The cfDNA could also serve as a new parameter in addition to the more well-known prognostic factors (nodes status, CEA levels, microsatellite stability status, KRAS/BRAF mutation status, resectability of metastatic disease, poor tumor grade, and hepatic tumor burden) to determine the prognosis of CRC patients in order to make a decision regarding the chemotherapeutic treatment. Several studies show that high ctDNA levels correlate with lower median progression-free survival (mPFS) and median overall survival (mOS)[34]. In particular, Messaoudi SE et al. have evaluated 97 cases of mCRC demonstrating that the ctDNA may be an independent prognostic factor ($p = 0.034$) and that the mutation load and the level of ctDNA fragmentation in KRAS/BRAF mutant patients inversely correlate with OS, highlighting differences up to 10 months[35]. Also using qPCR to compare levels of KRAS mutation found in the primary tumor and plasma could be assumed as an independent factor for PFS ($p = 0.002$) and OS ($p = 0.001$)[36]. CfDNA assessment could play a decisive role in cancer strategy in the next future, becoming a permanent part of a standard care protocol. A noble goal would be also the use ctDNA testing for early diagnosis of CRC. Most common screening methods used in clinical practice, such as colonoscopy, are indeed invasive and expensive procedures even if they ensure a good level of early diagnosis. Several studies have therefore investigated if it might be possible to detect mutations in the main genes involved in CRC pathogenesis directly from stool or blood, and nevertheless the results are still inconsistent. Today, the use of the liquid biopsy and in particular of ctDNA would also appear to be promising in this setting, since its levels could be positively correlated with the CRC (ROC: 0.709) in patients with the positive occult blood, although the method is still not able to intercept the precancerous lesions[37].

1.4 Rationale and Objectives

The prognostic value of the cfDNA was studied by Karen-Lise G. Spindler in a 2015 case-control study, in which the plasma levels of cfDNA were compared before the pharmacological treatment of patients with mCRC with cfDNA levels of healthy controls. This study showed that concentrations of cfDNA are higher in patients with CRC rather than in healthy controls. In addition, cfDNA concentrations of patients with CRC were stratified into quartiles and a progressively lower OS was observed from the lower quartile cfDNA levels to higher quartile. The prognostic reliability of the cfDNA has been compared with that of the current reference biomarker, ie the CEA. The overall evaluation of the studies shows that the cfDNA reflects the entity of the "tumor burden" in a superior way compared to the CEA, thus constituting a better prognostic factor[38, 39]. Recently, Fan et. al evaluated the prognostic role of ctDNA at the end of a systematic review of the data in the literature, correlating it with DFS and OS[17]. Many of the studies taken into consideration included a blood sample to be taken before treatment. The authors found that in 7 of 11 studies, ctDNA is an independent variable to estimate DFS, and in 8 studies out of 13 have identified that higher ctDNA concentrations represent a negative prognostic factor of OS. Sato et al. have highlighted the linear correlation between the concentration of ctDNA in the peripheral blood and the "tumor burden" in patients with non-metastatic CRC undergoing surgery[40]. The ctDNA, identified thanks to specific mutations of the single tumor for each patient, was present in lower concentrations than those measured in the post-operative sampling. Furthermore, the ctDNA assay in post-operative follow-up allows the evaluation of the minimal residual disease and is efficient in predicting disease recurrence[26]. Tie et al. explored the value of ctDNA as an early prognostic marker of response to first-line chemotherapy. Several blood samples were taken: before the therapy, 3 days after the first cycle and before the second cycle of therapy. Significant and early reductions in ctDNA concentrations were observed, capable of predicting the radiological response, which can be highlighted, however, 8-10 weeks after the start of treatment[29]. In the same way Garlan et al. observed that on the basis of ctDNA concentrations obtained immediately after the first treatment cycles, it was possible to distinguish patients as "good responders" and "bad responders", which respectively showed low and high values of ctDNA. The "good responders" reported a better response rate to therapy and longer PFS and OS, confirming the prognostic and predictive role of ctDNA[41].

The introduction of advanced technologies such as digital PCR (dPCR) and NGS has allowed a better and greater capacity for DNA sequencing, offering the possibility to amplify the small fraction of ctDNA from cfDNA and to derive from this information with important clinical implications. In mCRC, many studies have analyzed the correlation between mutations found in tumor tissue and those found in circulating tumor DNA. The mutation status of the KRAS gene was the most studied and a concordance between blood and tissue was identified ranging from 85% to 95% [42-45]. A meta-analysis conducted by Yi-Xin Hao et al. found, on the basis of the studies analyzed, a sensitivity of the research of KRAS mutations in the ctDNA of 67%, a specificity of 96% and a high diagnostic accuracy of the test, expressed by an AUC of 0.95[44]. An important study by the National Institute of Health (USA) focused on the analysis of

clinical features associated with cases of false negative patients (KRAS mutation found in the tissue but not in the plasma). These cases were more frequently associated with mucinous histology, young age, but especially at low levels of CEA[45]. The study found that a low tumor burden, indicated by low levels of CEA, is associated with low concentrations of ctDNA, such as to be not evaluable. Among the mutations investigated, there are those related to other genes involved in the EGFR cascade, such as NRAS, BRAF and PIK3CA[46-48]. Kidd-Sigal et al. they found a concordance between mutations in tumor tissue and ctDNA in BRAF and PIK3CA of 73.9% and 91.3%, respectively[49]. Beijer et al. they were among the first to propose the use of multigenic panels to be used in NGS to obtain an in-depth evaluation of tumor heterogeneity in colorectal carcinoma. This study tested the mutations in 9 of the most frequently mutated genes in colorectal carcinoma (BRAF, EGFR, KRAS, NRAS, PIK3CA, TP53, CTNNB1, FOXL2 and GNAS)[50]. Another important application of the mutation analysis of ctDNA is the possibility of conducting close and careful monitoring of the disease, both from a qualitative and quantitative point of view. In fact, sequencing of cfDNA samples detected at regular intervals allows the early detection of mutations that confer resistance to therapies with anti-EGFR monoclonal antibodies.

Currently, literature data indicate that mutations in genes involved in the EGFR pathway, particularly in KRAS, NRAS, BRAF, EGFR, MET and PIK3CA genes, can be identified, even 10 months before radiological progression[32], in the ctDNA of patients treated with anti-EGFR who do not respond to this therapy and progressively thereafter. A correlation study between the number of mutated copies of the KRAS gene and the response to therapy revealed an increase in the number of copies of KRAS mutated during disease progression, whereas, when anti-EGFR treatment was stopped, notes a decrease in the number of KRAS copies changed. This data suggests that patients who stop anti-EGFR therapy due to drug resistance may have the possibility of a re-challenge with anti-EGFR. Overall, these data indicate that with the analysis of ctDNA the mutational state of the tumor can be evaluated taking into account spatial and longitudinal heterogeneity[51]. Recently, the study of "tumor mutational load" or tumor mutational load, which involves the analysis of the number of mutations and the corresponding allelic frequency (variant allele frequency, VAF) has indicated the possibility of early intercepting the onset of recurrences or progression of illness, anticipating the outcome of the instrumental examinations.

Zhou et al. studied 6 patients with localized CRC who underwent surgery. A clear reduction in the allelic frequency and the number of non-synonymous somatic mutations in the post-operative phase was observed. In addition, 4 patients developed a disease recurrence, which these variables identified in advance with respect to CEA, Ca 19.9 and CT[52]. In 2017, in an innovative and still unique study in the field of liquid biopsy, the diagnostic accuracy was compared, in the identification of the KRAS gene mutation, the cfDNA and the exosomal dsDNA extracted from the plasma of patients with pancreatic ductal adenocarcinoma. The KRAS mutation in the exosomal dsDNA was detected in 66.7%, 80% and 85% of localized, locally advanced and metastatic carcinomas, respectively. In the same cohorts the KRAS mutation in the cfDNA was identified in 45.5%, 30.8% and 57.9%, respectively. The exosomal dsDNA

allows to identify mutations with greater accuracy than the cfDNA in localized and locally advanced carcinomas[53]. The biological explanation of this result seems to be linked to the origin of the two forms of DNA, since while exosomes are released into the blood by cells actively, the ctDNA represents an epiphenomena of the processes of necrosis and apoptosis that occur especially in the advanced stages of cancer.

Consequently, the aim of my research project is to evaluate the prognostic and / or predictive clinical utility of the CRC mutational status analysis using cfDNA and also exosomal DNA.

The analytical steps of our study are aimed at: the analysis of the concordance between the KRAS, NRAS and BRAF mutations found in the tissue and in the blood, in search of the prognostic and predictive value of the "mutation load" and finally the comparison between the information genetics carried by the cfDNA and the exosomal DNA of tumor origin, with a view to their use in clinical practice. I also will perform a systematic review and meta-analysis of literature data about the performance of laboratory techniques (PCR, BEAMing, NGS) to detect RAS mutation in mCRC using ctDNA.

CHAPTER 2

Materials and Methods

2.1 Patients' selection

A sample of 40 CRC patients was enrolled during chemotherapy with or without targeted agent at the U.O.C. of Medical Oncology of the University Hospital Policlinico Paolo Giaccone of Palermo, recruited on a voluntary basis after informed consent for participation in the study and for data processing.

One or more blood samples were collected in tubes containing EDTA before the beginning of chemotherapy infusion. Blood samples were collected initially at the time of the first instrumental evaluation and every 2-3 months. (Table 1)

2.2 Blood sample preparation

Plasma was separated from the cell fraction within one hour from the collection using two successive centrifugations: the first 1200 gx 10 minutes at 4 ° C to avoid cell lysis leading to the separation of the plasma from the corpuscular part of the blood and the second to 3000 gx 10 minutes at 4 ° C to eliminate cellular debris and red blood cells from plasma. Thereafter, the plasma was transferred into new 2 ml tubes and stored at -80 ° C until DNA was extracted from the sample.

2.3 Extraction of the circulating DNA

The extraction of the cfDNA was obtained from 1mL of plasma through the use of the commercial QIAamp® Circulating Nucleic Acid (Qiagen) kit which uses a vacuum pump to increase the extraction speed and efficiency. The procedure is easy and suitable for the simultaneous extraction of multiple samples. The extraction involves: a first phase of lysis, a purification phase through various washes, during which the DNA is retained by a membrane present in the column provided by the kit, and a final phase of elution in which the purified DNA is removed from the membrane by centrifugation after imbibition with the elution solution.

2.4 Quantization of the circulating DNA

Three μl of the extracted cfDNA were used to measure the concentration obtained. Quantization of free circulating DNA was performed using the Qubit™ dsDNA HS Assay Kit (Invitrogen, Life Technologies, CA, USA) in a Qubit 3.0 fluorometer (Invitrogen, Life Technologies, CA, USA). This is a very sensitive and specific test that can quantize even small concentrations of DNA (between 0.01-100 ng / μl). This quantization system allows to "measure" the emission of fluorescence through the use of a fluorophore, which specifically binds the double-stranded DNA (dsDNA).

2.5 Exosomes extraction

ExoEasy Maxi kit (Quiagen) was used to extract the exosomes from the plasma. This kit uses a column inside, a membrane able to specifically bind the entire spectrum of extracellular vesicles present in the sample, including also the exosomes. ExoEasy Maxi kit allows a rapid isolation of the exosomes using a quantity of 0.2-4 ml of plasma. It consists of a series of washes and centrifugations, which aim to remove from the membrane all that is not represented by extracellular vesicles. The last important elution step serves to detach the isolated vesicles from the membrane of the column and to keep them inside the tube.

2.6 Next Generation Sequencing (NGS) Analysis

The NGS analysis was conducted both on cfDNA and on isolated exosomes. In particular, the sequenced cfDNA samples, expressed with the CRC code, were 17 (2 for each patient except CRC-7). Among these 9 patients, 3 were chosen, of which the exosomes were isolated and the exosomal tumor DNA sequenced (CRC-3, CRC-13 and CRC-20). Also in this case, for each of the 3 patients, 2 plasma samples were used, taken at two different time points.

The NGS analysis was conducted on the IonS5 platform (ThermoFisher); The Ion AmpliSeq Cancer Hotspot Panel v2 (ThermoFisher) panel was used to perform the library preparation procedure. This panel contains 207 primer pairs in a single tube and allows to investigate hotspot regions of 50 oncogenes and tumor suppressor genes, with extensive coverage of KRAS, BRAF and EGFR genes.

The Ion AmpliSeq Library kit 2.0 (ThermoFisher) kit was used to prepare the libraries following the instructions given on the protocol.

The resulting libraries were then subjected to further amplification by PCR emulsion on the Ion OneTouch2™ (ThermoFisher) instrument. During this phase the library fragments are linked to the Ion Sphere Particles (ISP) thus forming the template which will then be used during the sequencing phase. At the end of the emulsion PCR, we proceed with the enrichment of the ISP using the Ion OneTouch™ ES instrument. The enriched ISPs are then loaded onto the Ion 530™ chip which is then subjected to

sequencing within the IonS5 instrument. During the run planning through the Torrent Suite™ Software, all the information that will be used in the following sequencing phase is provided (number of flows, barcodes and reference files). The Ion 520™ & Ion 530™ Kit-OT2 (ThermoFisher) kit was used to complete the above steps. The analysis of the data and the annotation of the variants was finally conducted through Ion Reporter Software™ by applying the AmpliSeq CHPv2 peripheral / CTC / CF DNA single sample workflow.

2.6 Statistical Analysis

Statistical analyzes were conducted using the MedCalc ver 14 software and SPSS software ver 19.0.

To compare the PFS and OS of patients with values of cfDNA less than the median and those with values higher than the median, a LogRank test was performed. Linear regression and the LogRank test were used to study the correlation between DSS, PFS and "tumor mutational load".

Numerous statistical analyses evaluated the distribution of factors such as the concentration of cfDNA and the "tumor mutational load" of patients grouped on dichotomous variables (eg patients with right-sided and left-sided colon cancer). The Student and Levene t tests were used provide my results.

3.1 cfDNA and prognostic role

Overall survival (OS) was evaluated. However, given the short duration of follow-up for some patients, the median was not reached after 5 patients out of 40 died (mOS 28,3 months) (Figure 6)

The same analysis for PFS was performed. The PFS was calculated between the first and second lines. Unlike the OS, in this case the median was reached and was equal to 14,5 months. Furthermore, it has been showed that patients with a concentration of cfDNA higher than the median value (0.47 ng/μl) had worse, statistically significant, PFS ($p=0,048$), according literature data. (Figure 7)

As regards cfDNA value and primitive neoplastic site, although patients with right-sided colon cancer reported a higher mean (0.704 ng/μl) than those with left-sided colon cancer (0.643 ng/μl), the difference was not statistically significant ($p>0,05$). (Figure 8)

A statistically significant correlation has been shown between cfDNA value and histological mucinous adenocarcinoma subtype. The mean concentrations of cfDNA in the mucinous adenocarcinoma and adenocarcinoma group was respectively 0,79 ng/μl and 0.57 ng/μl, statistically significant. (Figure 9)

3.2 cfDNA and predictive role

A possible cfDNA level difference has been showed in patients with disease progression (PD) if compared to patients who did not (PD was defined RECIST 1.1 criteria). The result obtained shows that the mean cfDNA concentrations of progressing patients (0.77 ng/μl) were statistically lower than the average of non-progressing patients (0.63 ng/μl) ($p=0,07$) (Figure 10), although a non-statistically significant difference was observed between the mean concentrations of cfDNA of patients receiving anti-EGFR (0.81 ng/μl) and the mean concentration patients treated with anti-VEGF (0.73 ng/μl) (Figure 11).

3.3 cfDNA and NGS

In the second part of the study, we selected nine patients with particular medical records (17 samples) to perform Next Generation Sequencing cfDNA. As shown in Tables 2 and 3, 2 samples of cfDNA collected from the blood of 9 patients were analyzed, at two different time points, with the exception of the CRC-7 code patient of which only one sample was available, for a total of 17 samples sequenced.

As shown in Table 3, a total of 309 mutations were discovered. Among these, 65 are non-synonymous somatic mutations, (61 have a single nucleotide substitution (SNV) and 4 are insertions/deletions (INDEL)). The remaining 244 are synonymous and intronic mutations.

The mean allelic frequency (variant allele frequency, VAF), expressed as a percentage, of non-synonymous somatic mutations found in the 17 samples was 38.3, with a VAF range of 1.62-100.

Figures 12 and 13 showed, for each gene involved, the number and frequency of non-synonymous mutations. The highest number of mutations was detected in the TP53, where the most frequent SNV was c.215C> G. Mutations in the APC, NRAS, PIK3CA and KRAS genes, notoriously involved in the Fearon and Vogelstein "adenoma-carcinoma sequence", showed a lower number of mutations. The KDR gene, which codes for VEGFR2, was found to be mutated in 3 metastatic patients treated with antiangiogenetics. The other genes, such as EGFR, PTEN and ERBB2 implicated in the CRC carcinogenic process by blocking the EGFR cascade, demonstrated a low frequency of mutations. Among the 17 samples analyzed in NGS, only in 2 was found the mutation in the KRAS gene, against the 5 mutations identified on the tissue. The correlation between KRAS mutations in the tissue and in the cfDNA is therefore 40%. NGS sequencing of the NRAS gene produced a different result since 2 mutations were detected in the CRC-3D and CRC-10A samples. However, no mutation of NRAS was detected on tumor tissue of both patients. At the same time, the NRAS Q61K mutation of the CRC-4 sample was not confirmed by liquid biopsy. Tissue mutation of the BRAF V600E gene of the CRC-3 sample was not detected in the cfDNA and overall the NGS analysis did not allow to detect mutations in the BRAF gene in any of the samples analyzed.

3.4 Prognostic role of tumor mutational load

Among the 17 samples analyzed in NGS, only in 2 samples a mutation in the KRAS gene was found respect to the 5 mutations identified on the tissue. The correlation between KRAS mutations in the tissue and in the cfDNA was therefore 40%. NGS sequencing of the NRAS gene produced a different result. Two mutations were detected in the CRC-3D and CRC-10A samples. However, no mutation of NRAS was detected on the tumor tissue of both patients. Mutation of the CRC-4 was not confirmed by liquid biopsy. The BRAF V600E gene of the CRC-3 sample was not detected in the cfDNA and overall the NGS analysis did not allow to detect mutations in the BRAF gene. Despite the small number of samples studied, it could be suggested a correlation between disease-specific survival

(DSS) and the number of non-synonymous mutations, statistically significant at the second time point. As shown in Figure 14, the number of non-synonymous mutations showed an inverse and significant correlation with the DSS ($p = 0.034$). Using the median value of non-synonymous mutations (3.5), it was found that patients with a number of non-synonymous mutations below the median value had a trend for better DSS, (LogRank test $p = 0.08$) (Figure 15). A similar result was obtained for PFS. The cumulative progression time reported a statistically significant increase ($p = 0.037$) as the number of total mutations decreases (Figure 16). However, also in this case, by subdividing this parameter according to the median value (17.5 mutations), the result was not statistically significant (LogRank test $p = 0.0896$) (Figure 17), probably due to the small number samples.

3.5 Exosomal tumor DNA NGS sequencing and comparison with cfDNA sequencing

A further step of the study was the exosomal DNA NGS analysis in comparison with the sequencing of cfDNA. Plasma exosomes samples were collected from 3 CRC patients (CRC-3; CRC-13; CRC-20), at 2 different time points (T1 and T2). Exosomal DNA and cfDNA were quantized and sequenced from the same blood sample. An interesting result emerged from the sequencing of the exosomal DNA of the CRC-20 sample.

As shown in Figure 18, 2 blood samples were collected from CRC-20 (the first at the third cycle and the second at the seventh CAPOX q-21 adjuvant cycle). Over a period of 6 months from the start of the treatment, an extensive lymph node and metastatic spread recurrence was CT evaluated and PET confirmed. Both the CEA and cfDNA values showed a trend that is consistent with the patient's clinical history, in fact a CEA 100-fold increase and a cfDNA 2-fold were observed.

The sequencing of the cfDNA extracted from the patient's blood to the first and second time points revealed a number of 5 and 8 non-synonymous somatic mutations respectively. The KRAS gene mutation was detected in the T2 sample, but not T1. In addition to mutation in the KRAS gene, functionally significant mutations were found in other genes such as: PIK3CA and ERBB2 (linked to KRAS and the EGFR pathway) and NOTCH1 (whose activity is able to suppress the Wnt expression even when the degradation of the β catenin by the APC complex is disabled by the mutation in the latter gene). The same analysis was performed on the exosomal DNA: at T1 17 missense mutations were identified, with a high average allelic frequency (VAF = 81). At T2 the result was not significant, probably for technique reasons. However, T1 exosomal DNA result could explain this case-report. I reported the KRAS mutation appearance in the T1 exosomal DNA according to neoplastic tissue mutational status. Interestingly, KRAS gene mutation was detected in cfDNA only at T2. Among the other genes, other mutations have been identified, in PIK3CA, ERBB4, SMARCB1 and SMAD4. SMAD4 gene mutation could assume a considerable role because of its prognostic role CRC. Finally, From the comparison with cfDNA, it would seem that the exosomes should be object of more in-depth studies as they could be able to highlight RAS mutations earlier than cfDNA.

Diagnostic Accuracy of DNA Analysis Techniques

Extracted from:

“Circulating tumor DNA for the detection of RAS mutations in metastatic colorectal cancer: a sistematic review of literature and meta-analysis”

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Under submission

4.1 Introduction

Several randomized trials have shown that the addition of molecularly targeted drugs (monoclonal antibodies, moAbs) as anti-vascular endothelial growth factor (anti-VEGF) and anti-Epidermal growth factor receptor (anti-EGFR) to conventional chemotherapy is able to improve the outcome of metastatic colorectal (mCRC) patients both in terms of progression-free survival (PFS) and overall survival (OS). In particular, retrospective analyzes carried out on the same studies have shown that a tissue-based mutation in one of the RAS family gene in a more comprehensive evaluation of RAS, so-called expanded RAS (KRAS exons 2,3,4 and NRAS exons 2,3,4), can more precisely select patients for an anti-EGFR therapy rather than KRAS codons 12 and 13 testing alone[54-57]. As a consequence, RAS testing is considered able to negatively predict the efficacy of anti EGFR moAbs in mCRC patients (representing about 55% of whole group)[58]. Therefore, tumor tissue

genotyping has become the routine approach in the management of these patients because of its ability to guide through the best first-line options[59, 60]. Nowadays, deeper understanding of the mechanisms linked to tumor heterogeneity allows us to know that the tumor is made up of a multitude of cellular clones with their proper genomic structure (spatial heterogeneity). Moreover, neoplastic clones acquire resistance to the selective pharmacological pressure through the expression of certain factors able to keep them alive in a harmful environment (spatial heterogeneity)[61-63]. Consequently, the monitoring of acquired resistances represents a fundamental step to establish the best timing for interrupting drug administration because of its ineffectiveness and its load of side effects. Furthermore, tissue biopsy although its ability to provide useful genomic information, remains burdened by various limitations related above all to its invasiveness, difficult reproducibility over time and poor representativeness of the entire tumor mass[64]. Numerous reports have recently shown how the liquid biopsy used in oncology field can be a potential new alternative to traditional tissue biopsy, thanks to its wealth of genetic material as circulating tumor DNA (ctDNA), circulating-free DNA (cfDNA), plasma exosomes and circulating microRNAs[19]. The advantages that have been reported, for liquid biopsy, are mainly related to its easy repeatability and the possibility of avoiding the selection bias related to tumor heterogeneity. Non-small cell lung cancer was one of the most studied tumors on this topic. Recently, the results from a large randomized controlled trial (AURA3) showed that the T790M mutation analysis performed by tissue or plasma through ctDNA detection in EGFR mutated NSCLC patients progressing after a first generation EGFR tyrosine-kinase inhibitor (TKI) has the same clinical value in terms of prediction of gains in PFS, OS as well as response to osimertinib, a third generation EGFR TKI, if compared to a platinum-pemetrexed regimen[65]. As these data were considered so consistent that the main international scientific societies (NCCN, ESMO, EMA) recommend the evaluation of the T790M mutation status on blood ctDNA as the first option compared to the traditional evaluation of tumor tissue biopsy[66]. Our recent work has recently systematically evaluated the performance of T790M analysis on blood ctDNA compared to the standard tissue in NSCLC demonstrating high specificity and sensitivity[67]. To date, however, the diagnostic accuracy of the RAS test on blood ctDNA in mCRC patients is still controversial. A recent meta-analysis provided an estimate of the prognostic relevance of cfDNA as a source of KRAS mutations by evaluating different conventional polymerase chain reaction (PCR) techniques, without considering the new methodologies now available such as the droplet digital PCR (ddPCR), BEAMing (Beads, Emulsions, Amplification and Magnetics) and high throughput approaches such as next generation sequencing techniques (NGS)[42]. Therefore, the objective of this article is to do a systematic revision of trials comparing matched blood and tumor tissue to provide a precise estimate of the diagnostic accuracy of the RAS gene mutation on ctDNA, also assessing new generation technologies in mCRC patients[27, 43, 48, 49, 68-82].

.2 Materials and Methods

4.2.1 Search for clinical trials

We searched all published studies that reported the plasma specificity and sensitivity data of the RAS mutation (KRAS or KRAS/NRAS if available) tested by ctDNA. We selected trials using electronic databases such as Medline (Pubmed), EMBASE, Cochrane libraries published until December 2018. Only human studies have been selected. English language restriction was used. We have also explored sources of gray literature were as the abstracts presented at the American Society of Clinical Oncology (ASCO) and European Society of Medical Oncology (ESMO) and Gastrointestinal Cancer Symposium meetings. The clinicaltrials.gov web-site was also investigated as a source of unpublished data (www.clinicaltrials.gov).

4.2.2 Selection Criteria

To select clinical trials we used the following criteria: 1) patients with histologically confirmed diagnosis of advanced CRC; 2) studies that contain RAS mutational evaluation (KRAS or KRAS / NRAS) in tumor tissue and matched plasma; 3) studies that included the sensitivity and specificity value of the RAS mutation tested by ctDNA using traditional (PCR) or new generation technique (ddPCR, BEAMing, NGS). The ongoing trials were excluded. In case of trial with several follow-up, the most updated trial was selected. The trials that did not include both the specificity and sensitivity values of the detection of blood RAS mutation by ctDNA were excluded.

4.2.3 Data Extraction

Data extraction and assessment were performed by two authors differently (A.G and S.T.). Disagreements were solved by discussion with a third author (A.R.). The following data were extracted from the selected studies: name of the first author, author nation, number of patients, year of publication, type of blood sample (plasma or serum) name of the journal, method of detection (ddPCR, BEAMing or NGS), evaluated gene (KRAS or KRAS/NRAS), true positive (TP), true negative (TN), false positive (FP) and false negative (FN) rates. The meta-analysis was drawn up according to the PRISMA - guidelines for reporting of systematic reviews[83].

4.2.4 Quality Assessment

The analysis of the overall quality of the studies included in the meta-analysis was conducted by two different investigators (A.G. and S.T) according to the QUASAD-2 tool (quality assessment of diagnostic accuracy studies 2) designed for the diagnostic accuracy analysis[84]. The quality assessment included four areas: patient selection, index test, reference standard, and flow and timing. For each study we declared "YES" as a low risk of bias and "NO" as a high risk of bias. We declared as "unclear" if the data were not sufficient for a precise judgment. Furthermore, two authors (A.G. and S.T.) evaluated the risk of selective outcome reporting bias and any disagreements were resolved by consensus.

4.2.5 Statistical Analysis

We extracted the data as TP (True positive), TN (True negative), FP(False positive), FN (False negative) and sample size included in each study about the status of RAS mutation on tumor tissue, considered as a gold standard, and on ctDNA considered as an experimental group (mutations in KRAS or KRAS/NRAS found in both tissue and liquid biopsy). We have considered: TP as the number of patients with RAS mutation found in both tissue and liquid biopsy; TN as the number of patients with RAS mutation not found either in the tissue or in liquid biopsy; FP as the number of patients with RAS mutation not found in the tissue but found in liquid biopsy; FN as the number of patients with positive tissue biopsy and negative liquid biopsy. These values have been calculated: specificity, sensitivity, concordance, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and the respective 95% confidence intervals (95% CI). The sensitivity was calculated as the ratio between TP and the sum of TP and FN x100 $[TP / (TP + FN) \times 100]$. The specificity was calculated as the ratio between TN and the sum of TN and FP x100 $[TN / (TN + FP) \times 100]$. The concordance was calculated as $[(TP + TN) / (TP + FN + TN + FP)] \times 100$. PLR was calculated as: $sensitivity / (1 - specificity)$ and represents the likelihood that a positive liquid biopsy for a RAS mutation can be found in a patient with a positive tissue biopsy for RAS mutation. NLR was calculated as: $(1 - sensitivity) / specificity$ and represents the likelihood that a negative liquid biopsy for a RAS mutation can be found in a patient with a negative tissue biopsy for RAS mutation. DOR was calculated as the ratio $(TP \times TN) / (FN \times FP)$ [85]. This measure expresses the magnitude of the odd that a patient is a carrier of a tissue mutation of RAS in the case of positive liquid biopsy or negative liquid biopsy for a RAS mutation. The sample sizes of the included studies were used as weight to pool specificity, sensitivity, PPV and NPV. PLR, NLR and DOR were pooled using the DerSimonian Laird method (random effects model) to consider variance between studies[83]. Furthermore, we have elaborated a summary receiver operating characteristics (sROC) curve and calculated its area under the curve (AUC). A subgroup analysis was also performed with regard to the new generation detection techniques included in this meta-analysis (BEAMing, PCR and NGS). To assess the presence of a possible bias linked to the threshold effect, the Spearman correlation was used between the sensitivity logit and the 1-specificity logit. The threshold effect was considered significant if p value <0.05. The other sources of heterogeneity not dependent on the threshold effect were calculated using the Cochrane Q test (as the weighted sum of squared differences between individual and the index of inconsistency (I^2) that indicate the percentage of variance between the studies that is explained by heterogeneity and not by chance. Heterogeneity was considered significant if the p value of the Q test was <0.05 or if I^2 value > 50%. A meta-regression was also performed to identify other sources of heterogeneity and the publication bias test to evaluate any other bias among the studies was included in our analysis. The related Funnel plot (for visual inspection) and the Egger test were produced, considered significant if P-value <0.05. All analyzes were conducted using the MetaDisc statistical software (version 1.4)[84].The publication bias calculation was performed using the MetaEssential software[85].

4.3 Results

4.3.1 Characteristics of eligible studies

Our research of literature trials found a total of 410 articles published until December 2018. Of these, nineteen met the inclusion criteria and were therefore included in our pooled analysis with a total of 1810 patients (Figure 19). The study by Sefrioui et al [80] evaluated the RAS mutation status by both digital chip PCR and cast PCR and the data were reported as two independent studies, with a total of twenty items included in our pooled analysis. All these studies included patients with histologically confirmed mCRC with collection of tissue matched to blood (plasma or serum) for the ctDNA RAS mutation detection. The most used technology was NGS (8/20 studies). Six studies used the PCR technique (6/20) and six studies used BEAMing (6/20). Regarding the positivity thresholds, although 2/20 studies did not report a precise threshold, 18/20 studies expressed it as mutant allele concentration (copies / ml) or mutant allele fraction (%). All the studies in which underwent the pooled analysis included the values of specificity and sensitivity of ctDNA for the detection of RAS mutation (KRAS or KRAS / NRAS) compared with the tumor tissue considered as the gold standard. Sensitivity ranged from 50,0 to 100%, while specificity ranged from 66,7 to 100% among the different studies. The main characteristics of the included trials have been summarized in table 4.

4.3.2 Diagnostic Accuracy analysis

The pooled sensitivity and specificity of ctDNA in our analysis were 0,83 (95% CI: 0,80 to 0,85) and 0,91 (95% CI: 0,89 to 0,93) respectively (Figure 20). The pooled PPV and NPV of the ctDNA were 0,87 (95% CI: 0,81 to 0,92) and 0,87 (95% CI: 0,82 to 0,92), respectively. PLR was 8,20 (95% CI: 5,16 to 13,02) and NLR was 0,22 (95% CI: 0,16 to 0,30). The pooled DOR was 50,86 (95% CI: 26,15 to 98,76) and the AUC of the sROC curve was 0,94 (Figure 21). A subgroup analysis was conducted to evaluate if different methods could influence the diagnostic accuracy of the ctDNA and their results are shown in figures 22 and 23 and summarized in table 5.

4.3.3 Threshold effect and heterogeneity

To evaluate the presence of bias associated to the threshold effect we calculated the correlation coefficient of Spearman and the relative p value. Spearman's coefficient was -0,186 (p value = 0,433) and therefore not significantly related to bias. Despite this, however, a source of high heterogeneity that was not dependent on the threshold effect was found and therefore a meta-regression was performed which results demonstrated how the different methods of ctDNA analysis were not associated with heterogeneity.

4.3.4 Quality Analysis and Publication bias

The presence of publication bias was investigated using the MetaEssential software. The Funnel plot did not reflect particular asymmetries and also the Egger test is not significant ($p = 0,89$) (Figure 24). The overall quality of the trials included in the meta-analysis was assessed as good by using the QUADAS-2 tool (Figure 25).

CHAPTER 5

Discussion

In recent years, numerous scientific reports have evaluated the diagnostic, prognostic, predictive and monitoring role of cfDNA and ctDNA. Both are promising non-invasive biomarkers that have contributed to the recent rise of liquid biopsy[17, 46]. Despite this study, some limitations including the impossibility of recording the OS data can be explained by the smallness of the sample, by the scarcity of the follow-up and by the impossibility sometimes to perform the blood collection at the correct time-point. Anyway, obtained results provides a reasonable rationale to further continue the study, increasing the sample number and the average follow-up to validate liquid biopsy role.

One of the best important application of cfDNA concentration as a biomarker for monitoring the response to therapy was the evaluation of the variations during chemotherapy infusion. CfDNA concentrations on the basis of radiological progression showed a statistically significant trend, although the type of targeted therapy did not. However, I'm waiting to reach a number of samples for each patient that allows a more accurate dynamic evaluation of the tumor and mutation load of CRC.

About the site of the primary tumor, my result suggests higher cfDNA value in patients with right colon CRC patients, although the is not statistically significant.

Of note, a statistically significant result was obtained for histological type. In fact, the mucinous forms of CRC showed significantly higher levels of cfDNA than those obtained from patients with classic adenocarcinoma suggesting greater tumor activity linked to mucinous type to explain its already known prognostic role.

The limited number of available blood samples did not allow a precise dynamic monitoring of the genetic profile. However, analyzing the results obtained, interesting cases have emerged. First, some tissue mutations have not been confirmed by the cfDNA. Secondly, the cfDNA allowed to highlight mutations not found in the tumor tissue (NRAS patient). In the first case, the non-concordance could be due to a limitation of the technique used (DNA extraction and sequencing). On the other hand, however, we must consider that most of the patients have undergone the resection of the primary tumor, as well as extreme variability of the metastases number and size. So, the different tumor burden could influence the amount of gene mutations found in cfDNA. In the second case, we can instead assume that the number of mutated copies of the NRAS gene at the time of tissue

biopsy was not sufficient to detect it. In contrast, over time, the increase in the number of mutated copies of the NRAS gene was seen in the cfDNA.

Regarding "mutational load" predictive and prognostic role, different trends were observed between the number of mutations, the VAF and the survival estimates. This encouraging result surely pushes us to expand the sample number and to seek further confirmations on the reliability of the mutational tumor load as a promising biomarker in CRC.

Moreover, there is increasing evidence that the intercellular communication mediated by exosomes plays an important role in the process of carcinogenesis. In fact, they intervene in angiogenesis, premetastatic niche development and immune system tumor escape[86, 87]. The case under report has shown that the exosomal DNA is able to intercept the KRAS gene mutation before cfDNA gene. Clinically, it could allow to define disease recurrence and the secondary resistance phases. From the analysis of the exosomal DNA cargo, other mutations have also emerged among which the SMAD4 gene, which has a prognostic role CRC.

Biologically, the above mentioned diagnostic anticipation could be explained by the ability of the exosomes, equipped with a membrane, to transport and protect the load of proteins and nucleic acids within them. On the contrary, since the cfDNA in the blood is subjected to continuous degradation and denaturing processes, a longer time is required for mutated genes to exceed the sensitivity limit of the NGS. In addition to sensitivity, a further important difference between the cfDNA and the exosomal DNA is related to specificity. In fact, ctDNA, which derives from the processes of necrosis and apoptosis of neoplastic cells, constitutes only a small fraction of the cfDNA. Vice versa, the exosomes are actively released from the cancer cells and their content seems to be fundamental in the tumor economy, so the sequencing of their DNA allows a specific assessment of the genetic picture of the neoplasm. Consequently, these reasons justifies the need to know the diagnostic accuracy of the technology available today to remove the limits of genomic analysis and to improve the cost/effectiveness ratio.

Although our study compared the two sources of DNA in only one patient, I hypothesize that the sequencing of exosomal DNA can guarantee a true "real time" evaluation of tumor dynamics, thus constituting a highly reliable non-invasive biomarker in the disease course.

In light of this, the most fascinating perspective is undoubtedly that of using a Next Generation Sequencing panel that allows to study the mutational status of single genes related to the pathogenesis of CRC and "tumor mutational load". Although the results obtained are promising, further research and studies represent a clinical need to validate and standardize the procedures used. In order to provide an effective estimate of the specificity and sensitivity of the technologies used for the assessment of circulating tumor DNA, I performed a systematic review of the literature with meta-analysis of the results in collaboration with other authors. Our meta-analysis includes 20 studies for a total of 1825 mCRC patients in which the tissue matched paired with blood were available for the assessment of mutational status of RAS (KRAS or KRAS / NRAS) using ctDNA. The results show a good performance of ctDNA in terms of overall specificity (0,91) and sensitivity (0,83) for the RAS mutation if compared to tumor tissue considered as gold-standard. However, this analysis shows that about 20% of patients defined as negative by liquid biopsy were positive for tissue analysis. This could probably be

influenced by the techniques (see Figures 22-23 and table 5). However, our results reinforce even more the hypothesis that these tools should be used at the most opportune timing and in the most appropriate phase of disease course. Technologies such as quantitative PCR and NGS probably could be reserved to the first mutational assessment in an advanced disease phase, reserving other technologies such as BEAMing to the monitoring of already established genetic alterations. The early knowledge of the disease mutational profile is one of the most relevant clinical need, since it can have a considerable impact at different phases of natural history, such as diagnosis, first line guidance and dynamic monitoring for the detection of acquired resistance[88]. New generation technologies allow the analysis of multigenic panels and several patients at the same time, guaranteeing high performance standards and economic savings. Several articles have discussed the potential role of ctDNA in CRC by highlighting the detection of higher levels in more advanced stages of disease. It has also been hypothesized that elevated levels of ctDNA in CRC are correlated with poor outcome, as its primary post-surgery finding may suggest the presence of minimal residual disease and its potential role for the definition of actionable targets. As above mentioned, although fragmentary data, some evidence seems to suggest a potential role also in the monitoring of secondary resistances. Misale et al [89] showed for the first time in mCRC patients treated with anti EGFR (Panitumumab or Cetuximab) that to discover an amplification of KRAS through liquid biopsy was able to anticipate the radiological progression by 10 months with a sensitivity of 60% . Furthermore, Siravegna et al [51] demonstrated that the interruption of treatment with antiEGFR reduced the number of mutated KRAS clones allowing a reacquisition of the sensitivity to anti-EGFR agents. This information suggested novel rationales potentially useful for the management and continuum of mCRC care. Recently, Goldberg et al[88] recently proposed that the ctDNA evaluated by liquid biopsy can become an optimal tool for deciding between the strategy of of cetuximab continuum or rechallenge in order to extend the number of available therapeutic options and optimize the therapeutic strategy. Determinants factors are ctDNA power of detection and the optimal timing for rechallenge to an anti-EGFR agent. Rossini et al [49] described the results of the CRICKET study in which 27 mCRC wild type RAS / BRAF patients with acquired resistance during the first line anti-EGFR regimen were treated with Irinotecan-Cetuximab as rechallange strategy. In this experience, the ctDNA evaluated by ddPCR and NGS at the rechallange baseline was able to predict a greater efficacy of Irinotecan-Cetuximab in wild type ctDNA RAS patients compared to RAS mutant ctDNA in terms of progression-free survival (3.9 vs 1.9 mos ; Hazard Ratio 0.48), identifying RAS mutations in 9/25 among patients evaluable by liquid biopsy (48%). These data show how easy access to liquid biopsy can offer a possible solution to the inter- and intratumor heterogeneity that characterize the onset of drug resistance. Recent reports suggest that RAS profile may differ between primary and metastatic site in about 20% of cases. Therefore, it could be assumed that a portion of the false negatives and false positives rates of ctDNA RAS could be explained by the site of the disease examined (primary vs liver or lung metastases) since recent experiences suggest that the emerging mutations, not found in the primary tumor, may instead be shown in the metastasis site or in blood[90] Although, CRC are highly heterogeneous, their intratumor heterogeneity features are less studied than other cancer types. Recent findings show that intratumor heterogeneity can be tested by single-cell sequencing; the single-cell genome sequencing permit

quantitative characterization of both single nucleotide variations and somatic copy number variations in individual tumor cells[91]. Recently, a Nature Opinion article suggests that the main operative unit of a cancer is the genetically and epigenetically modified single cell. The single-cell analysis can allow to clarify the intra-tumoral genetic heterogeneity and cancer genome evolution in order to develop new tools able to provide robust interpretation of the mechanisms related to diagnosis, tumor recurrence and the functioning of new generation molecular target agents and the development of secondary resistances modifying cancer patient management[92, 93]. Despite these potentialities, the use of liquid biopsy in routine clinical practice has historically been burdened by still substantial costs, heterogeneity in methodology and results from studies or in different threshold values that could be influenced by the noise caused by normal tissue cfDNA. For these reasons, the level of evidence to date is not yet considered as high to recommend the use of ctDNA RAS testing in routine clinical practice. Our meta-analysis therefore provides for the first time a clear and comprehensive evidence of feasibility and good performance of ctDNA RAS testing in mCRC patients providing a strong rationale for its development into randomized clinical trials to be validated prospectively.

Figures and Tables

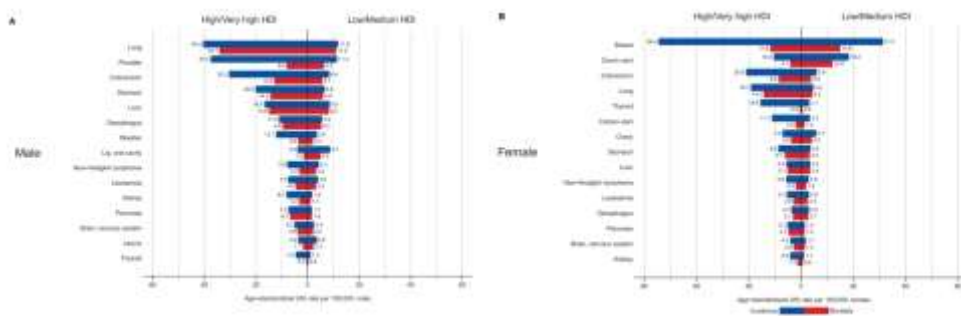


Fig.1: Bar Charts of Incidence and Mortality Age-Standardized Rates in High/Very-High Human Development Index (HDI) Regions Versus Low/Medium HDI Regions Among (A) Men and (B) Women in 2018. The 15 most common cancers world (W) in 2018 are shown in descending order of the overall age-standardized rate for both sexes combined. Source: GLOBOCAN 2018.

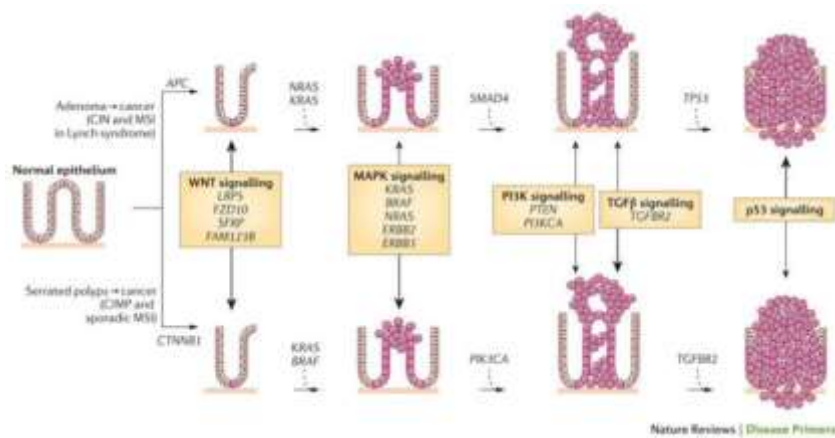


Fig.2: The normal epithelium can turn into adenocarcinoma passing through the adenoma (top) or the sessile serrated polyp (bottom). The two pathways share different types of genetic and epigenetic alterations, but some genes are exclusive of one path rather than the other.

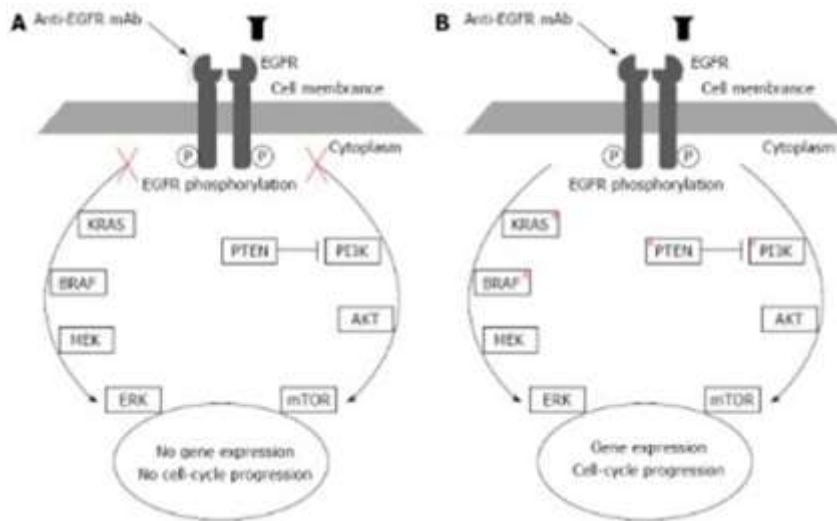


Fig.3: Representation of the EGFR pathway in response to treatment with anti-EGFR monoclonal antibodies in "all-wild-type" (A) and mutated (B) patients.

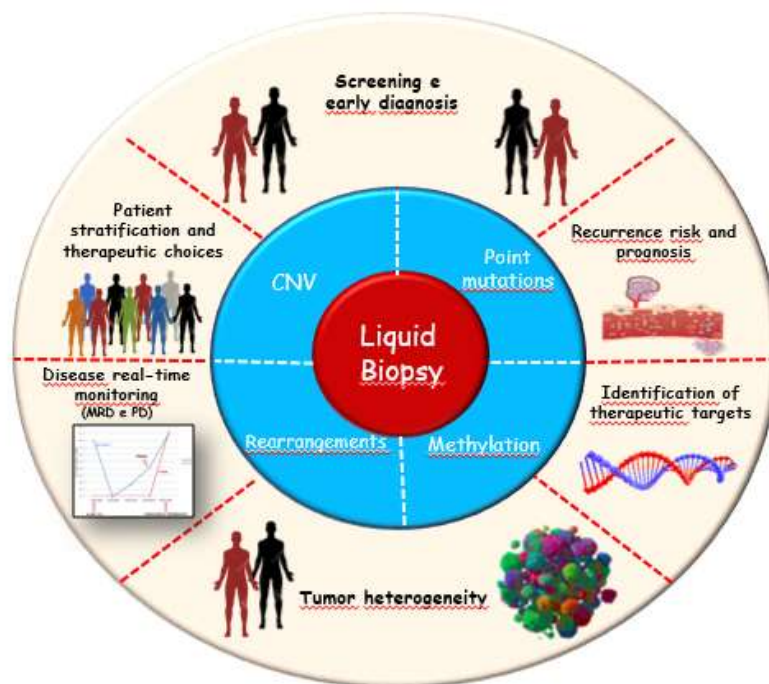


Fig.4: Possible implications of liquid biopsy in colorectal cancer (CRC) management

Tissue Biopsy vs. Liquid Biopsy

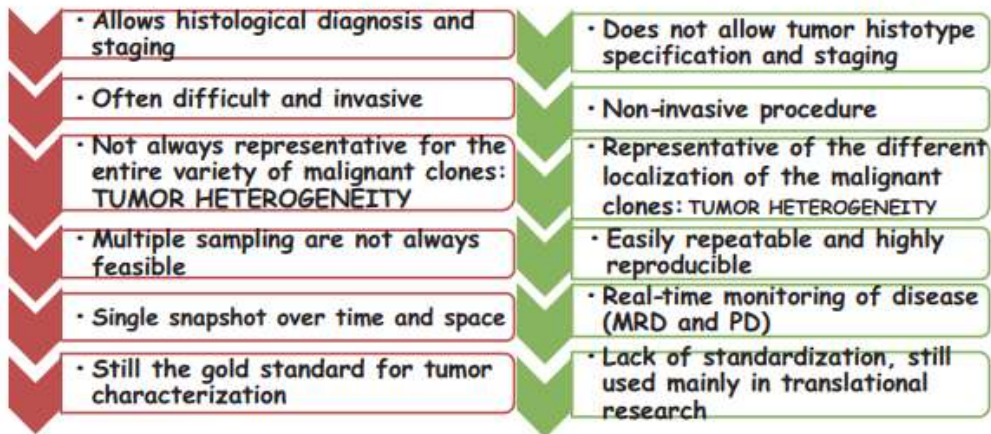


Fig.4: Direct comparison between Tissue biopsy and Liquid biopsy

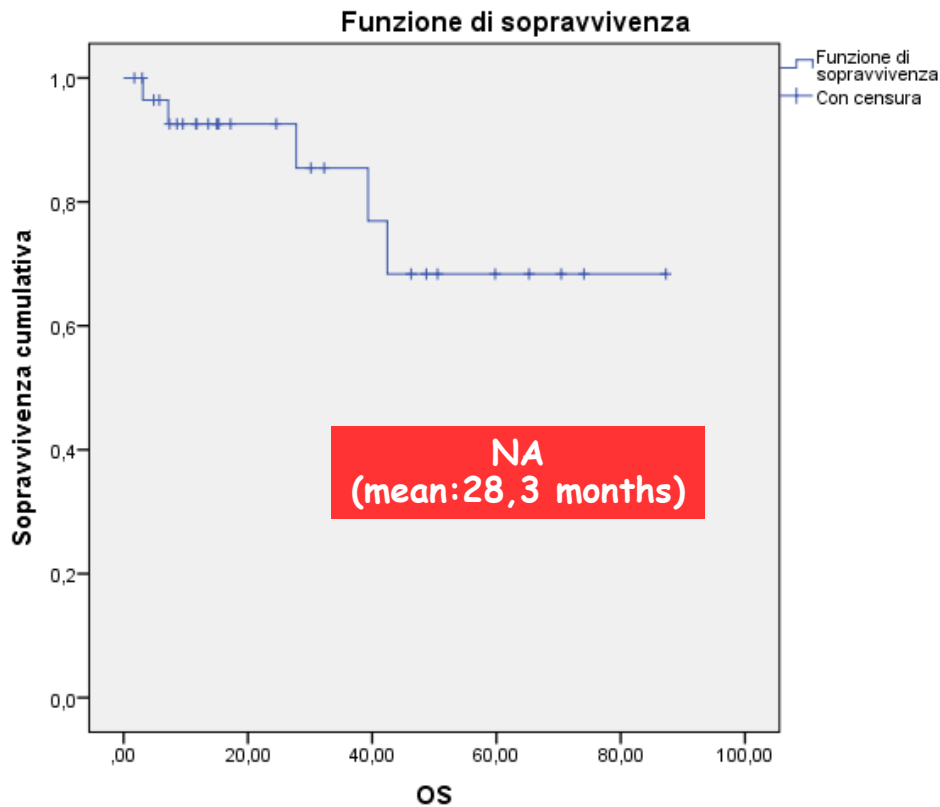


Fig.5: Median Overall Survival (OS) (NA: Not applicable)

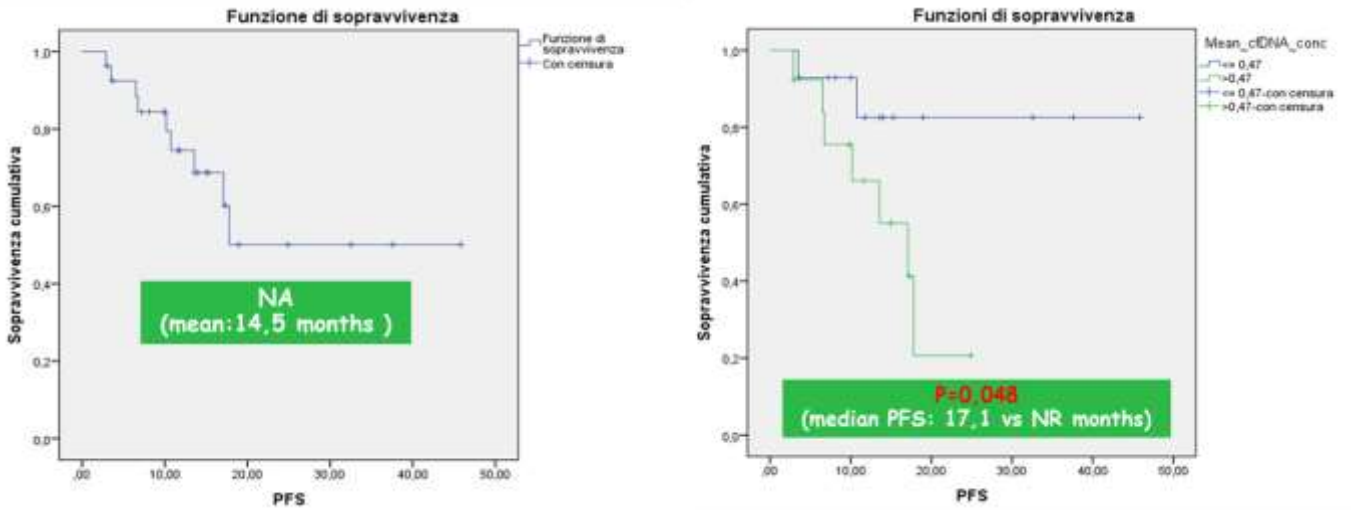


Fig.6: Median PFS (A) and PFS according to median [cfDNA] (NA: Not applicable)

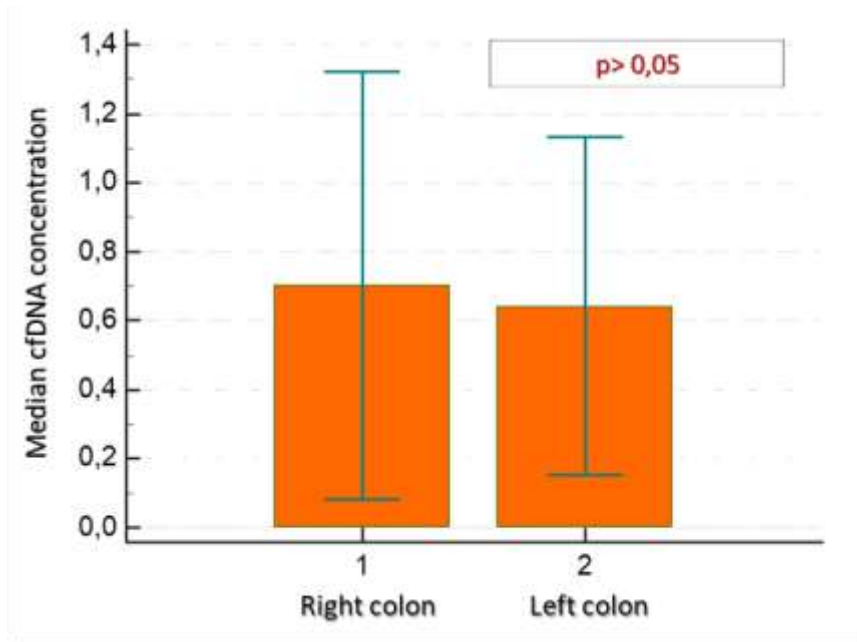


Fig.7: [cfDNA] and primary mCRC sidedness

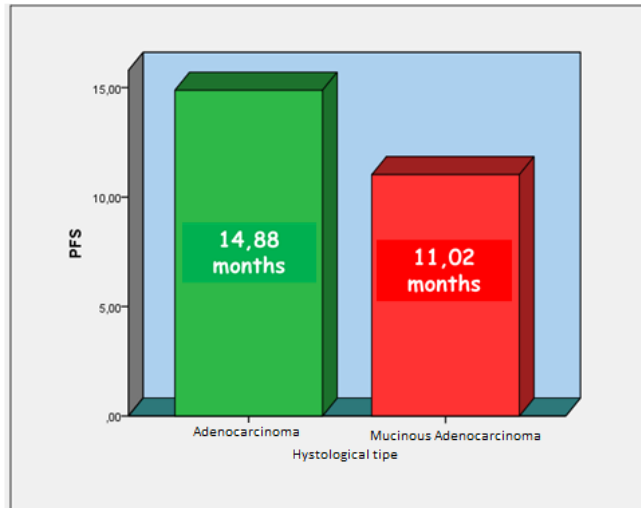
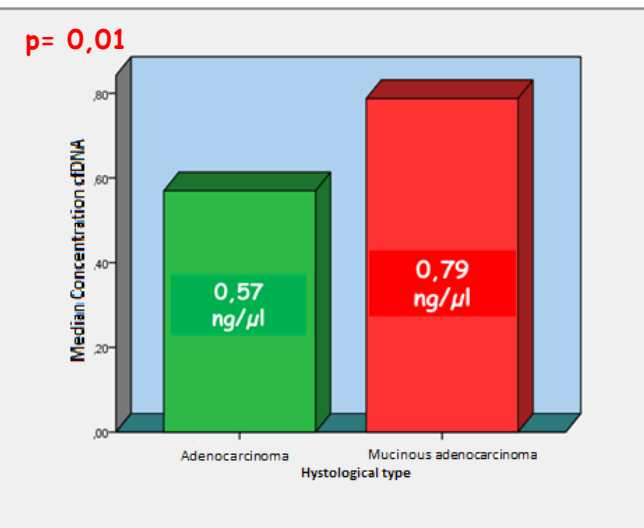


Fig.8: [cfDNA] and PFS and hystological type

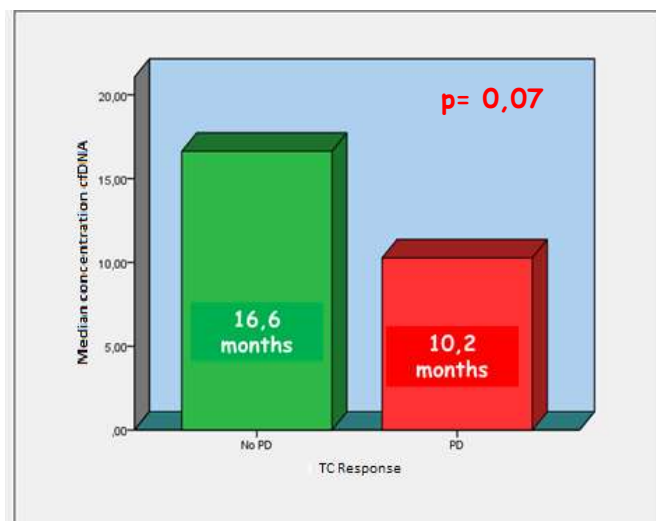
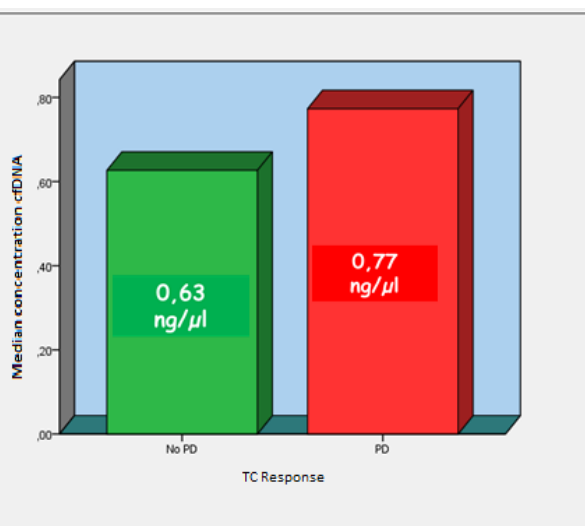


Fig.10: [cfDNA] and PFS and TC response

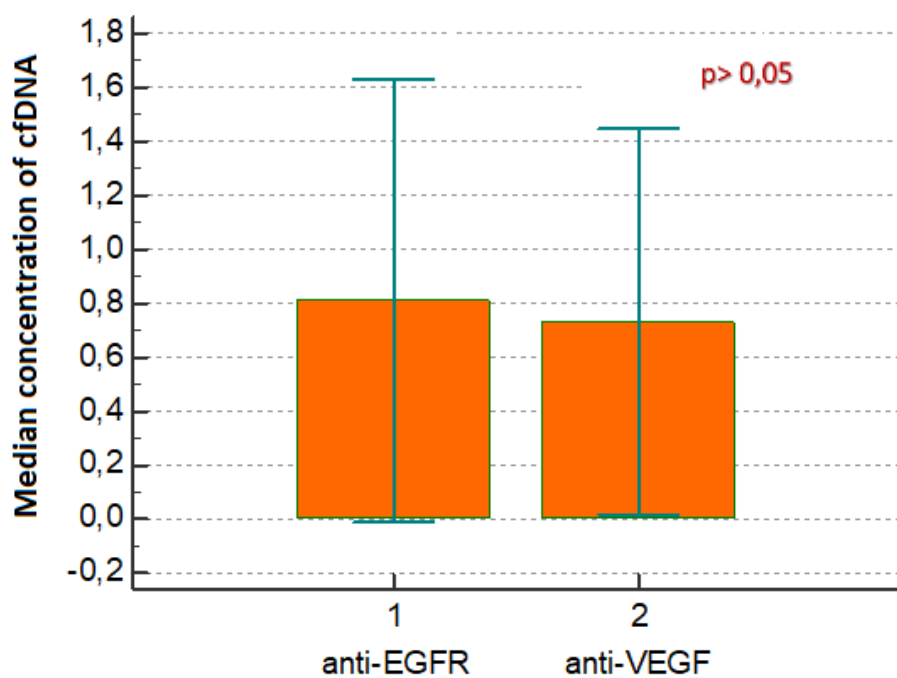


Fig.9: [cfDNA] and targeted agents

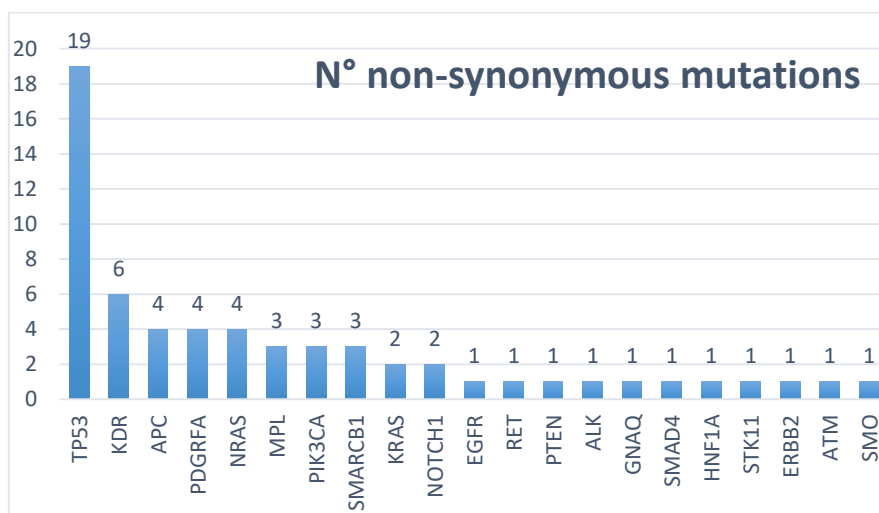


Fig.102: Non-synonymous mutations (N°)

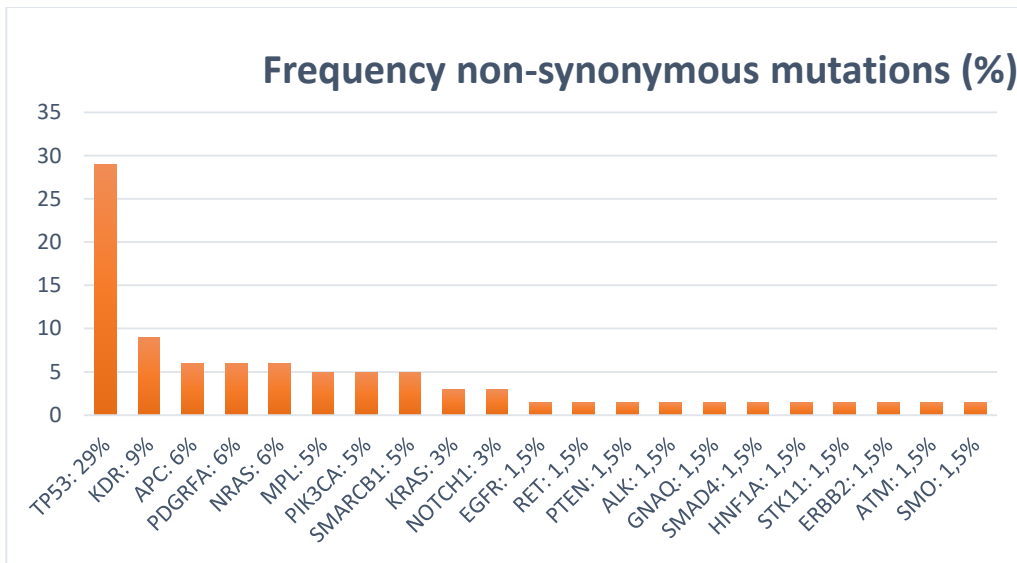


Fig.13: Non-synonymous mutations (%)

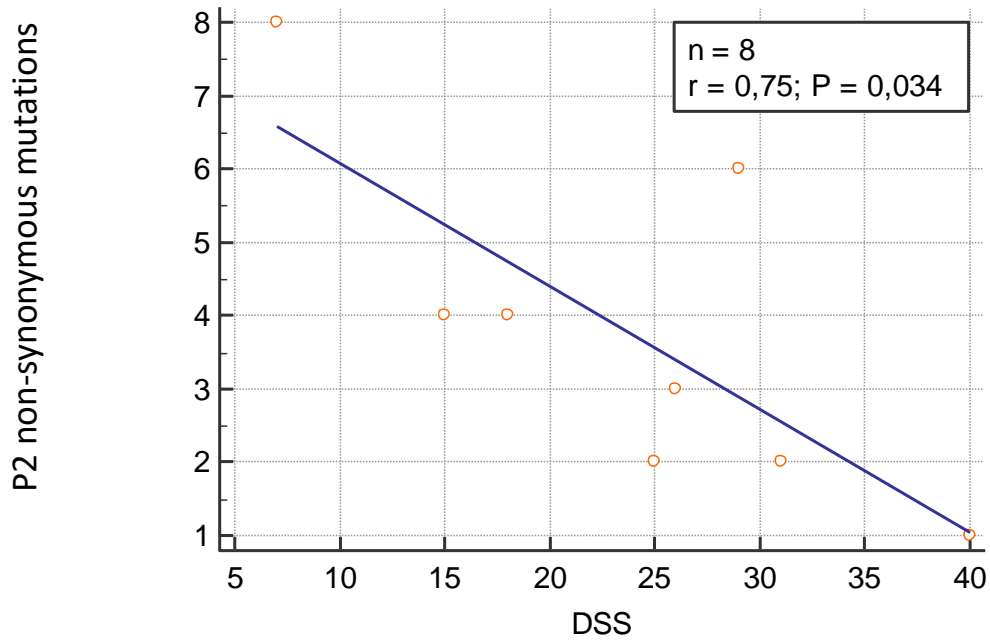


Fig.14: Correlation between sample collection 2 (P2) and disease-specific survival (DSS)

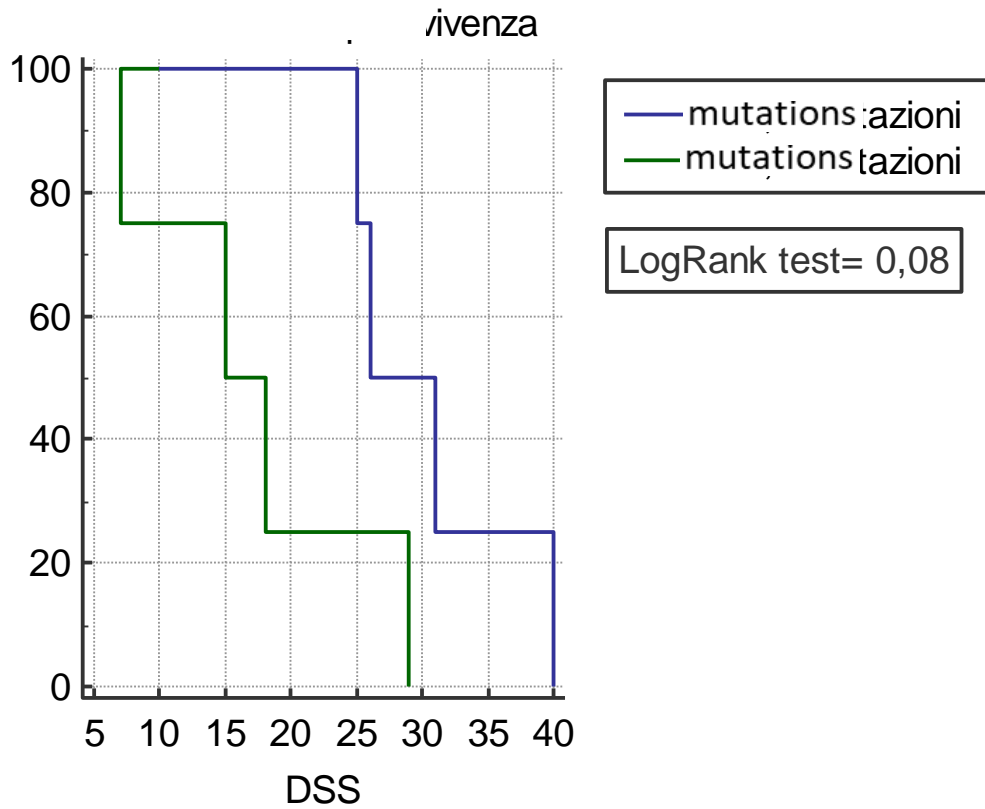


Fig.15: Disease-specific survival (DSS) and non-synonymous mutations

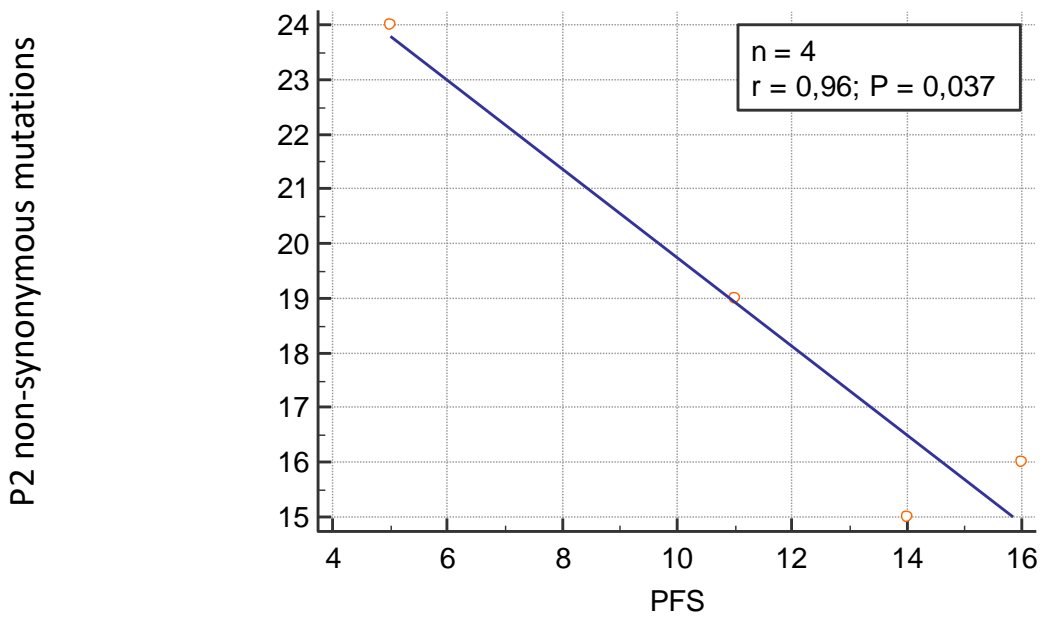


Fig.16: Correlation between sample collection 2 (P2) and progression-free survival (PFS)

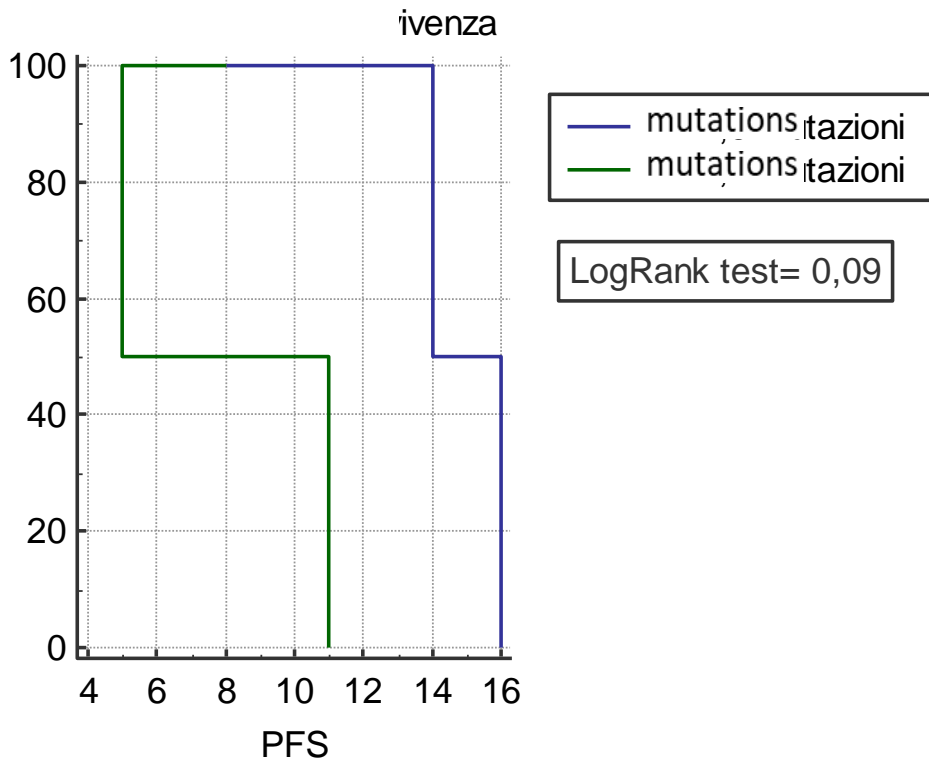


Fig.17: Progression-free survival (PFS) and non-synonymous mutations

68 y.o. – Male – Right CRC

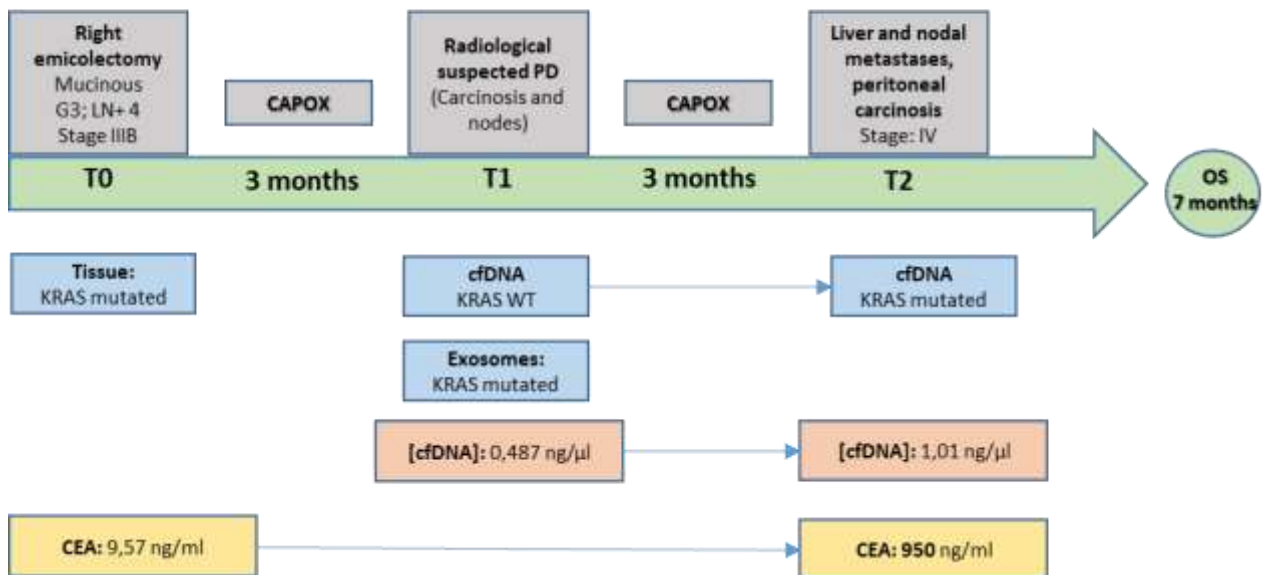


Fig.18: CRC-20 case report



Fig.19: Flow chart of trials included in Meta-Analysis

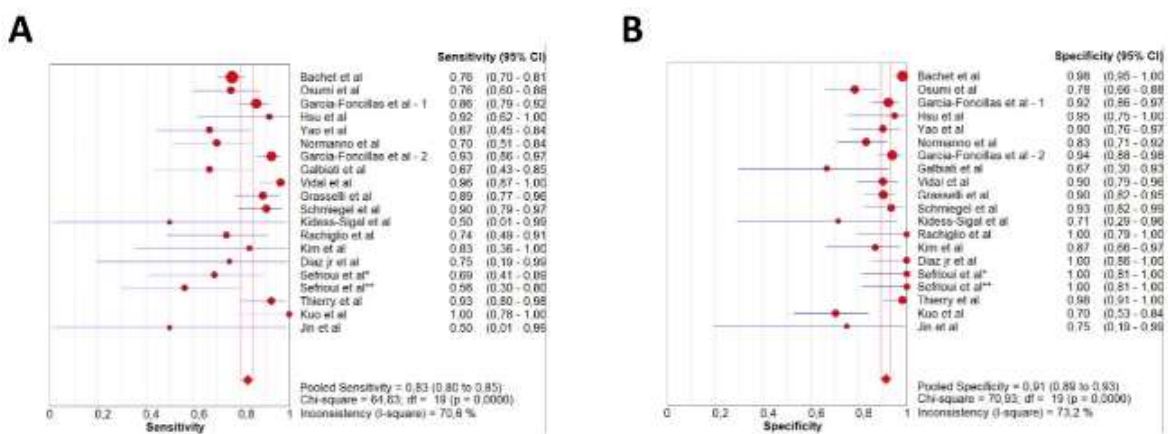


Fig.2011: Forest plots of sensitivity (A) and specificity (B) of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) mutation.

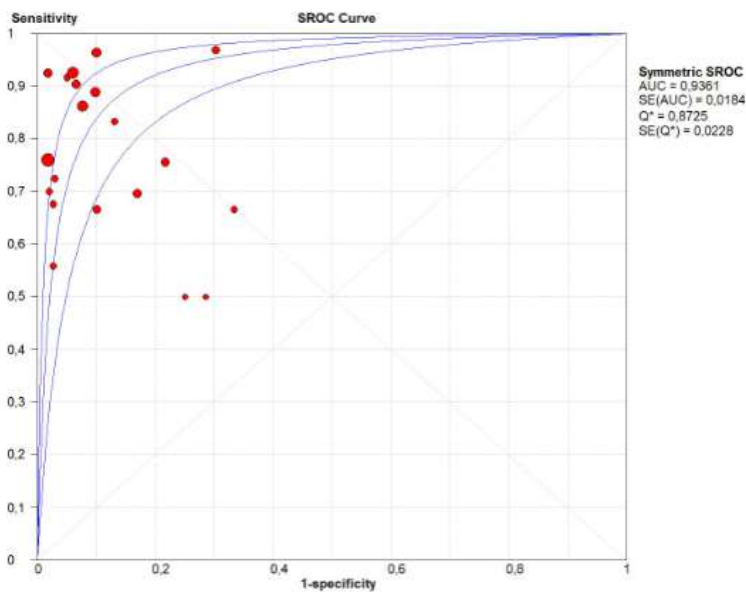


Fig.2112: SROC curve of ctDNA for detection of RAS (KRAS or KRAS/NRAS) mutation

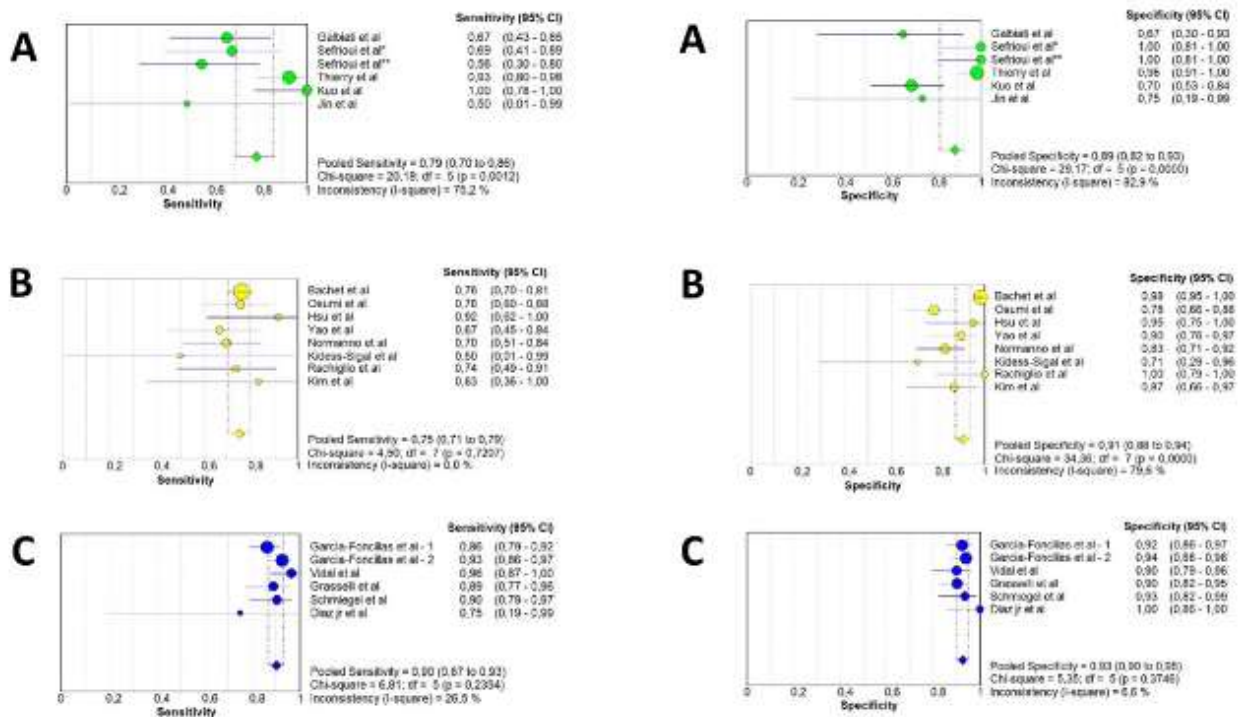


Fig.22: Forest plots of sensitivity and specificity of ctDNA for the detection of RAS (KRAS or KRAS/NRAS) according to the different diagnostic methods: (A) PCR; (B) NGS; (C) BEAMing.

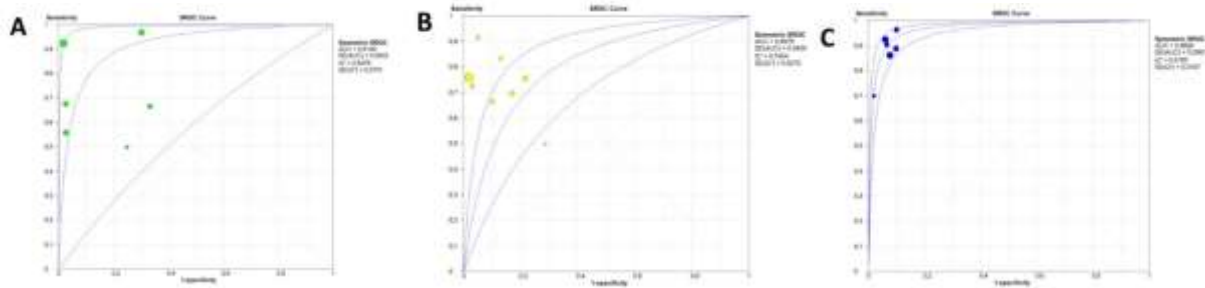


Fig.23: SROC curve of ctDNA for detection of RAS (KRAS or KRAS/NRAS) mutation according to the different diagnostic methods: (A) PCR; (B) NGS; (C) BEAMing

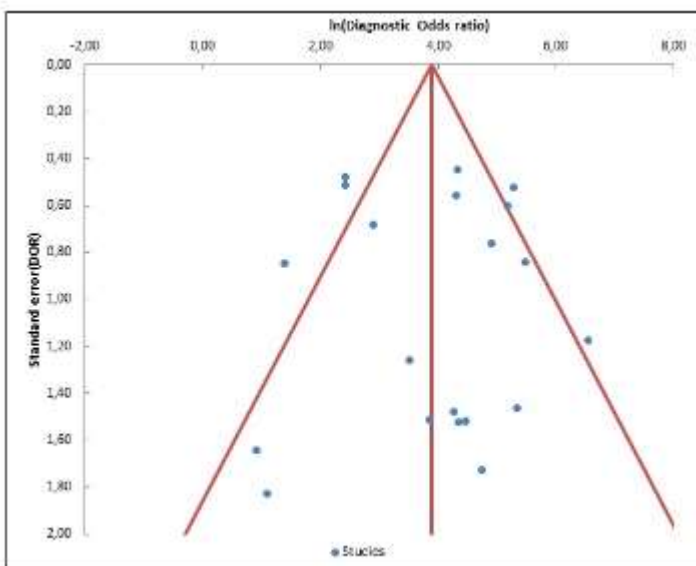
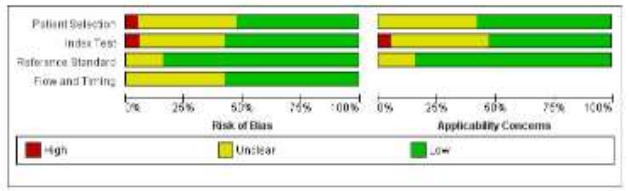


Fig.24: Funnel plot of diagnostic odds ratio (DOR) for ctDNA detection of RAS (KRAS or KRAS/NRAS) mutation. Each circle represents a study and the vertical line represents the pooled effect estimate.

A



B



Fig.25: Quality assessment of included trials by QUADAS-2 tool (A: Summary; B: Graph).

N° Patients	40
Mean Age	62 (48-81)
Sex	
Female	17 (42,5%)
Male	23 (57,5%)
Primary tumor sidedness	
Right colon	16 (40%)
Left colon	24 (60%)
Neoplastic nodes	
>10	32 (80%)
≤ 10	5 (12,5%)
NA	3 (7,5%)
Grading	
G1	2 (5%)
G2	23 (57,5%)
G3	10 (25%)
NA	5 (12,5%)
Hystological type	
Adenocarcinoma	29 (72,5%)
Mucinous Adenok	11 (27,5%)
Diagnosis TNM stage	
IV	22 (55%)
III	12 (25%)
II	4 (5%)
I	2 (5%)
CT Lines	
Adjuvant	2 (5%)
I linea	20 (50%)
II linea	8 (20%)
III linea	10 (25%)
CT Type	
CT	7 (17,5%)
CT+Anti-VEGF	29 (72,5%)
CT+Anti-EGFR	4 (10%)
Survival status	
Alive	35 (87,5%)
Died	5 (12,5%)

Table 1: Clinical features of patients included in my research project

N° Patients	9
Mean age	66 (52-75)
Sex	
Female	6 (67%)
Male	3 (33%)
Primary tumor sidedness	
Right colon	5 (56%)
Left colon	4 (44%)
Neoplastic nodes	
< 10	1 (11%)
≥ 10	7 (78%)
NA	1 (11%)
Grading	
G1	0
G2	3 (33%)
G3	5 (56%)
NA	1 (11%)
Hystological type	
Adenocarcinoma	5 (56%)
Mucinous Adenok	4 (44%)
Diagnosis TNM stage	
IV	8 (89%)
III	1 (11%)
Linea di terapia	
Adjuvant	1 (11%)
I line	3 (33%)
II line	4 (44%)
III line	1 (11%)
Survival status	
Alive	8 (89%)
Dead	1 (11%)

Table 2: Clinical features of patients included in NGS

Code	[cfDNA]	number of non-synonymous mutations	total number of mutations	Mean VAS% (non-synonymous)	number of cycles P1-P2	CT	PFS	DSS	OS
CRC-1	0.423	5	21	22.604%	18	SD	14	40	No
	0.180	1	15	96.25%					
	2.79	2	16	46.085%					
CRC-3	0.428	6	22	17.8%	21	PD		29	No
	0.867	2	14	50.19%					
CRC-4	1.11	3	16	34.4%	20	PD	16	26	No
	0.523	6	17	48.31%					
CRC-7	0.362	4	19	15.4%	NA	NA	14	26	No
CRC-10	0.813	2	15	73.62%	5	SD	NA	25	No
	0.374	5	18	31.55%					
CRC-13	0.225	4	16	39.55%	18	PD	NA	18	No
	0.253	3	17	67.43%					
CRC-16	0.747	4	19	50.37%	6	PR	11	15	No
	0.487	5	24	48.61%					
CRC-20	1.01	8	24	15.7%	4	PD	5	7	SI
	0.293	3	20	49.46%					
CRC-26	0.626	2	16	72.95%	13	SD	NA	31	No
Totale		65	309	38.3%					

Table 3: NGS results. CT: Computed tomography response; SD: Stable Disease; PR: Partial Response; PD: Progression disease; DSS: Disease-specific survival; PFS: Progressiion-free survival, OS: Overall survival; P1: Blood sample collection ; P2: Blood sample collection 2; NA: Not Applicable

Study (reference)	Country	Number of patients	Sample	Detection Method	Gene	Sensitivity n (%)	Specificity n (%)	Concordance n (%)	NPV n (%)	PPV n (%)
Bachet et al	France	412	Plasma	NGS	KRAS, NRAS	184/412 (44.7%)	267/570 (46.8%)	35/142 (24.6%)	267/229 (116.6%)	184/187 (98.4%)
Quint et al	Spain	101	Plasma	NGS	KRAS, NRAS	32/101 (31.7%)	47/68 (69.1%)	30/121 (24.8%)	47/57 (82.6%)	32/44 (72.7%)
Garcia-Arriola et al (1)	Spain	126	Plasma	Beaming	KRAS, NRAS	135/130 (96.2%)	97/135 (92.4%)	212/238 (89%)	97/115 (84.3%)	135/120 (95.8%)
Hsu et al	Taiwan	32	Plasma	NGS	KRAS	12/32 (37.5%)	18/20 (90%)	30/32 (93.8%)	18/32 (56.3%)	12/11 (90.9%)
Yao et al	China	78	Plasma	NGS	KRAS	18/78 (23.1%)	38/50 (76%)	32/84 (38.1%)	38/44 (86.4%)	18/20 (90%)
Benmarouf et al	Italy	90	Plasma	NGS	KRAS, NRAS	15/90 (16.7%)	48/59 (81.4%)	31/52 (59.6%)	48/59 (81.4%)	15/20 (75%)
Garcia-Arriola et al (2)	Spain	128	Plasma	Beaming	KRAS, NRAS	132/120 (91.7%)	110/117 (94%)	212/238 (89.1%)	110/115 (95.7%)	132/128 (94.5%)
Gallati et al	Italy	30	Plasma	dPCR	KRAS	14/31 (45.2%)	6/9 (66.7%)	20/30 (66.7%)	6/13 (46.2%)	14/17 (82.4%)
Vidal et al	Spain	118	Plasma	Beaming	KRAS, NRAS	15/103 (14.6%)	54/62 (87.1%)	227/128 (178%)	54/68 (79.4%)	15/19 (78.9%)
Green et al	Spain	90	Plasma	Beaming	KRAS, NRAS	48/54 (88.9%)	84/92 (91.3%)	152/154 (98.7%)	84/89 (94.4%)	48/57 (84.2%)
Schmid et al	Germany	98	Plasma	Beaming	KRAS, NRAS	47/92 (51.1%)	43/48 (89.6%)	80/98 (81.6%)	43/48 (89.6%)	47/50 (94%)
Kido-Sugawara et al	USA	9	Plasma	NGS	KRAS	1/9 (11.1%)	9/7 (128.6%)	6/9 (66.7%)	9/10 (90%)	1/8 (12.5%)
Beaughnot et al	Italy	18	Plasma	NGS	KRAS, NRAS	14/18 (77.8%)	14/14 (100%)	30/31 (96.8%)	14/15 (93.3%)	14/14 (100%)
Kim et al	Korea	19	Plasma	NGS	KRAS	5/19 (26.3%)	15/13 (115.4%)	21/29 (72.4%)	15/15 (100%)	5/8 (62.5%)
Onoz et al	USA	18	Serum	Beaming	KRAS	9/18 (50%)	24/24 (100%)	21/18 (116.7%)	24/25 (96%)	9/10 (90%)
Sethou et al	France	34	Plasma	dPCR (1)	KRAS	11/38 (28.9%)	18/18 (100%)	28/34 (82.4%)	18/13 (138.5%)	11/11 (100%)
				dPCR (2)		9/14 (64.3%)	18/18 (100%)	27/34 (79.4%)	18/13 (138.5%)	9/9 (100%)
Thierry et al	France	95	Plasma	in-line PCR	KRAS	57/95 (59.9%)	86/97 (88.7%)	92/97 (94.8%)	86/99 (86.9%)	57/66 (86.4%)
Kwon et al	Taiwan	52	Plasma	Flow-PCR	KRAS	15/52 (28.8%)	26/37 (70.3%)	41/52 (78.8%)	26/34 (76.5%)	15/16 (93.8%)
Im et al	Korea	8	Plasma	qPCR + PCR	KRAS	1/8 (12.5%)	7/7 (100%)	6/8 (75%)	7/7 (100%)	1/8 (12.5%)

Table 4: Characteristics of Trials Included in the Meta-Analysis. PCR: Polymerase chain reaction; dPCR: digital-PCR; NGS: next-generation sequencing; BEAMing: Beads, Emulsions, Amplification and Magnetics; CI: confidence intervals; PPV: positive predictive value; NPV: negative predictive value.

	Number of patients	Sensitivity (95%CI)	Specificity (95%CI)	DOR (95%CI)	NLR (95%CI)	PLR (95%CI)	AUC
All Studies	1810	0,83 (0,80 – 0,85)	0,91 (0,89 – 0,93)	51,35 (26,85 – 98,18)	0,22 (0,16 – 0,30)	8,27 (5,25 – 13,03)	0,94
NGS	786	0,75 (0,71 – 0,80)	0,91 (0,88 – 0,94)	28,28 (10 – 79,94)	0,27 (0,23 – 0,32)	7,39 (3,2 – 17,13)	0,81
BEAMing	807	0,90 (0,87 – 0,93)	0,93 (0,90 – 0,95)	113,26 (68,72 – 186,66)	0,11 (0,08 – 0,17)	11,41 (8,23 – 15,82)	0,97
PCR	217	0,79 (0,70 – 0,86)	0,89 (0,82 – 0,93)	36,6 (5,6 – 239,55)	0,29 (0,14 – 0,63)	6,72 (1,84 – 24,54)	0,91

Table 5: Meta-Analysis Results. PCR: Polymerase chain reaction; NGS: next-generation sequencing; BEAMing: Beads, Emulsions, Amplification and Magnetics; CI: confidence intervals; PLR: positive likelihood ratio; NLR: negative likelihood ratio; DOR: diagnostic odds ratio; AUC: area under curve.

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Scientific Products in agreements with the Research Project

1. **Galvano A.**, Taverna S., Castiglia M., Passiglia F., Fulfaro F., Tagliaferri P., Bazan V., Russo A. Circulating tumor DNA for the detection of RAS mutations in metastatic colorectal cancer: a systematic review of literature and meta-analysis. Submitted and under review.
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3. **One book chapter (Liquid Biopsy in CRC – Springer NY edition 2017 - Prof. M.Peeters)**

Scientific Products - ISI

1. Bronte G, Silvestris N, Castiglia M, **Galvano A**, Passiglia F, Sortino G, Cicero G, Rolfo C, Peeters M, Bazan V, Fanale D, Giordano A, Russo A. New findings on primary and acquired resistance to anti-EGFR therapy in metastatic colorectal cancer: do all roads lead to RAS? *Oncotarget.* 2015 Sep 22;6(28):24780-96. doi: 10.18632/oncotarget.4959. Review. PubMed PMID: 26318427; PubMed Central PMCID: PMC4694794.
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NON ISI

- 1. One book Chapter** (Cardioncology: Principles, prevention and management – Elsevier 2017 Prof. A.Russo)
- 2. One book Chapter** (Colorectal liver metastases – Prof. M.Peeters)
- 3.** AIOM I numeri del cancro (Anal Neoplasm – AIOM 2017 – Prof. A.Russo, Dott. C.Aschele)
- 4.** AIOM Guidelines (Cardioprotezione – AIOM 2017 – Prof. A.Russo)
- 5. One book Chapter** (Liquid Biopsy in Prostate Pancer – Springer NY edition 2017 – Prof. C. Rolfo)
- 6. One book Chapter** (Cardiovascular Complications in Cancer Therapy – Springer 2018 – Prof. A.Russo, Prof. G.Novo)
- 7. One book Chapter in press** (Colorectal Cancer – Springer 2018 – Prof. A.Russo)

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