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Predictive biomarkers in colorectal adenocarcinoma – a Brazilian perspective on current and future practices

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Abstract

Predictive biomarkers of response to therapy are fundamental for the personalized therapeutic management of patients with colorectal carcinoma (CRC). The main predictive biomarkers related to CRC are the mismatch repair proteins/microsatellite instability status (MMR/MSI status), RAS/RAF mutation status and HER2 status. We discuss the scenarios in which these biomarkers are used and address different aspects that may affect the evaluation of each biomarker. We also address the increasingly recognized importance of circulating tumor DNA (ctDNA) testing in the management of stage II-III CRC as well as the role of the pathologist in this setting.

Introduction

Colorectal cancer (CRC) is the third most common cancer in Brazil among both men and women, excluding non-melanoma skin tumors. According to data from INCA, approximately 45,000 new cases are expected each year, with the highest incidence in the southeastern region of Brazil (Santos et al. 2023). These figures are similar to global projections, where it also ranks third in terms of incidence, with an estimated 1,9 million new cases per year (Bray et al. 2022). Generally, this type of tumor is diagnosed around the sixth and seventh decades of life. In recent years, diagnosis in younger age groups has become increasingly common, mainly due to modifiable factors such as lifestyle (Kim and Hanna 2023).

One of the main strategies for prevention and early detection of CRC is colonoscopy. This endoscopic examination not only allows for the diagnosis of lesions in early and asymptomatic stages but also enables the treatment of precursor lesions such as adenomatous polyps with dysplasia, which have a higher risk of malignancy.

Generally, the curative treatment of CRC is based on the surgical resection of the primary lesion and adjuvant chemotherapy, depending on the clinical and pathological staging. Stage II tumors, when necessary, receive adjuvant therapy with fluoropyrimidine mono-chemotherapy. When facing high-risk tumors like pT4 with adverse features, such as perineural or perivascular invasion, tumor perforation, or requiring emergency surgery with limited lymph node dissection, oncologists may opt for adjuvant treatment with fluoropyrimidine and oxaliplatin. However, this choice is subject to ongoing debate among specialists. In stage III tumors, polychemotherapy based on fluoropyrimidine and oxaliplatin is generally performed.

Apart from microsatellite instability testing, little has been altered in the adjuvant treatment and clinical follow-up of the patient based on tumor biomarkers.

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Typically, biomarkers are not incorporated into the pathologist's routine in the localized disease setting; instead, they are reserved for scenarios of recurrence or metastatic disease. However, with decreasing costs and wide availability, these biomarkers are increasingly requested earlier in clinical practice.

Generally, predictive markers in oncology are characteristics investigated in tumors that confer them with some type of different response to a proposed treatment. In addition to being responsible for histological diagnosis and pathological staging according to updated protocols, pathologists play a central role in the evaluation of predictive markers in CRC. The main predictive factors related to CRC are the mismatch repair proteins/microsatellite instability status (MMR/MSI status), HER2 status and *RAS/RAF* mutation status. These tests and the role of the pathologist will be discussed further.

MMR/MSI status

Importance for treatment decision making

Of all predictive markers in routine CRC, MMR/MSI status is currently the only predictive biomarker that alters the clinical practice of oncologists in adjuvant therapy (i.e., preventive treatment of micrometastases after curative surgery for localized cases). Therefore, pathologists are increasingly being asked to perform MMR/MSI tests earlier in CRC diagnosis.

A large retrospective study that analyzed samples from patients included in three major clinical trials for CRC adjuvant therapy showed that those with microsatellite instability/deficiency in DNA repair enzymes did not benefit from receiving adjuvant chemotherapy regimens based on fluorouracil (5-FU) (Ribic et al. 2003). For patients with stage II CRC with a high risk of recurrence, where the main drug of choice for adjuvant therapy is 5-FU in monotherapy, knowing the repair enzyme status beforehand will often lead the oncologist to omit adjuvant treatment.

In addition to this scenario, a recently published phase 2 study showed that the treatment of localized disease can also be altered in rectal cancer specifically in the case of microsatellite stability (Cercek et al. 2022). This single-arm study included only MSI patients and offered Dostarlimab (anti-PD-1 immunotherapy) in a neoadjuvant regimen for 6 months for patients with stage II and III rectal cancer. In the initial trial protocol, chemoradiotherapy (CRT) and surgery were to be offered to patients who did not achieve complete clinical response. The primary objective was to analyze the rates of complete response after 12 months of patient follow-up. In this study, all 12 evaluated patients achieved complete clinical response only with the use of immunotherapy in the neoadjuvant setting, without the need for CRT or surgery. Although with a small sample size and immature follow-up results, the study shows such promising results that it can be incorporated into current clinical practice without the morbidity of the current treatments.

In the metastatic disease scenario, with the Food and Drug Administration (FDA) approval of Pembrolizumab (anti-PD-1 immunotherapy) as an agnostic treatment for patients with mismatch repair deficiency, this drug has become a viable therapeutic strategy in CRC. The publication of the KEYNOTE-177 trial confirmed the efficacy of anti-PD-1 immunotherapy in this specific population. In this phase 3 trial (Andre et al. 2020), patients with metastatic CRC and microsatellite instability who had not received prior treatment were randomized to receive pembrolizumab or standard chemotherapy based on 5-FU and anti-VEGF or anti-EGFR, as first-line treatment. As expected for this specific population, there was an improvement in progression-free survival rates in the group receiving immunotherapy (16 vs. 8 months in the standard regimen group), which was confirmed several years later with also an overall survival improvement (not assessed in the pembrolizumab group vs. 36 months in the standard group) (Diaz et al. 2022).

Methods for MMR/MSI status testing

Evaluation of MMR status by immunohistochemistry

Pathologists can assess the proficiency of the DNA repair system by both immunohistochemistry and molecular methods. Immunohistochemistry evaluates whether the protein expression of the main genes involved in DNA repair (MSH2, MSH6, MLH1, PMS2) is intact or not in the tumor tissue. These proteins exhibit nuclear staining that reflects their role in correcting DNA replication errors during the cell division process. A case is considered proficient (pMMR) if all four proteins are detected. Conversely, a case with a deficient repair system (dMMR) lacks at least one of the four proteins, (Chen and Frankel 2019) (Fig. 1).

False-positive or false-negative results that do not reflect the status of the DNA repair system can occur due to both technical and biological reasons. Technical reasons are the main cause of false-negative results, mainly due to pre-analytical fixation artifacts. In a study on the influence of pre-analytical factors on the accuracy of MMR/MSI tests in mucinous CRC, Malapelle et al. (Malapelle et al. 2020) found no impact of the year of the paraffin block of the sample on the quality of IHC. However, it was not reported whether the evaluated samples had been subjected to a controlled fixation time. Grillo et al. (Grillo et al. 2023) recently showed that both under-fixation (<20 h) and over-fixation (>90 h) impair the proper detection of MMR proteins by immunohistochemistry, affecting mainly MLH1 and PMS2. Larger samples more frequently presented substandard staining (patchiness and central artifact) when compared to

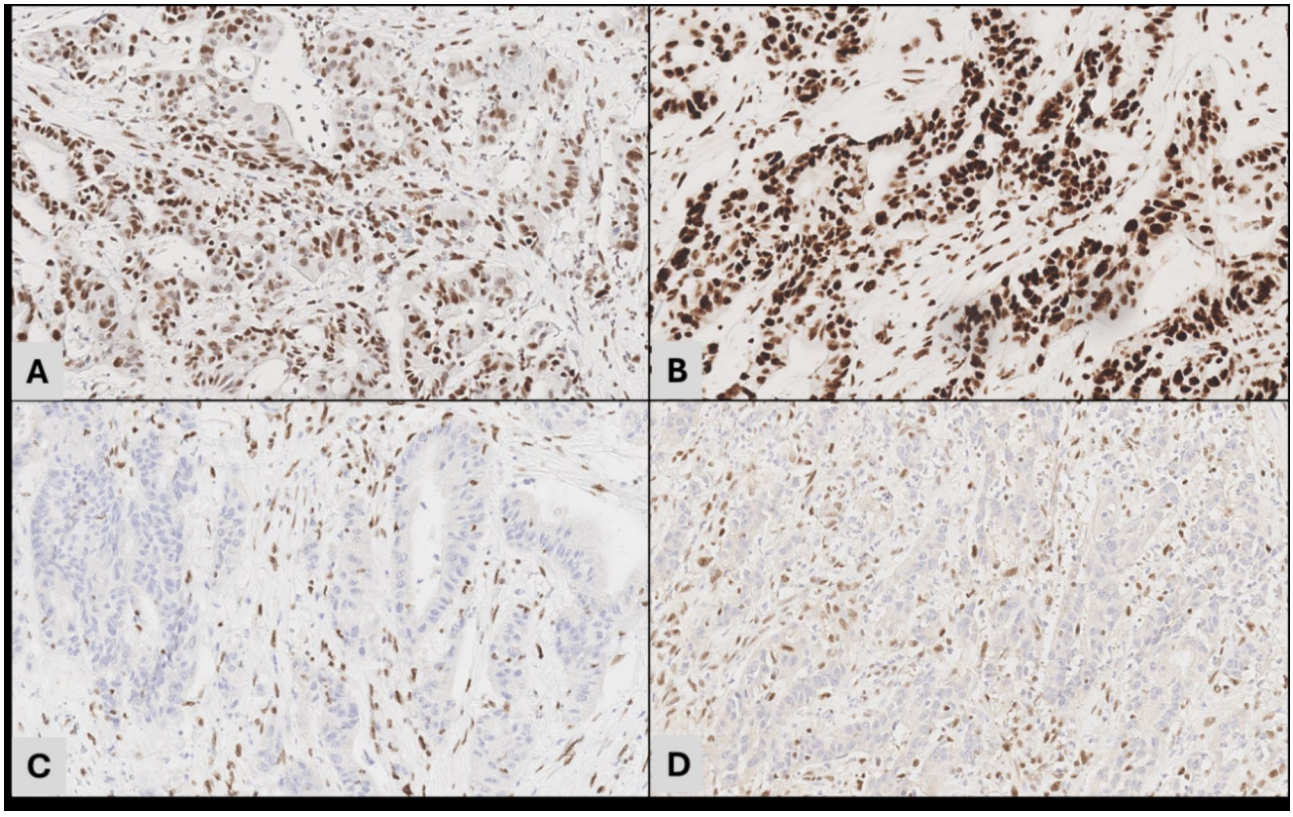

Fig. 1 Mismatch repair immunohistochemistry in colorectal cancer. In a proficient tumor, intact nuclear staining of MSH2, MSH6, MLH1 and PMS2 is expected. The case depicted shows retained expression of MSH2 (**a**) and MSH6 (**b**) and concomitant losses of MLH1 (**c**) and PMS2 (**d**) (i.e., a dMMR case). Staining in background stromal cells and lymphocytes serves as positive internal control and validates the quality of the immunohistochemical reaction. dMMR: deficient mismatch repair. Original magnification 200x

smaller samples. The formalin temperature (4 °C versus room temperature) was also compared in samples with standard fixation time (24–48 h), and it was observed that fixation at 4 °C resulted in fewer cases with inadequate reaction. Although the authors recommend controlling the fixation time (24 h) and using formalin at 4 °C to achieve better results in MMR IHC evaluation, for most pathology laboratories worldwide, the use of formalin at 4 °C is a difficult or impossible requirement to follow. In these cases, attention to fixation time and sample size is important to minimize the number of cases with inadequate testing. In the case of samples from surgical specimens with fixation artifacts, the analysis should prioritize the sample from the previous biopsy that established the CRC diagnosis.

Biological reasons are the cause of discordant results where a false-negative IHC result (i.e., retained expression of all four MMR proteins) is seen in cases with pathogenic missense or frameshift/truncation mutations resulting in a protein with altered functional activity, but which maintains the antigenic site recognized by the antibody during immunohistochemical reaction (Luchini et al. 2019).

Given an optimal IHC test, different expression patterns can be found in a dMMR tumor. The most common pattern involves the loss of the MSH2/MSH6 or MLH1/PMS2 dimer, or isolated loss of MSH6. A less common finding is the isolated loss of PMS2. Rarer findings involve isolated loss of MSH2 without concomitant loss of MSH6, isolated loss of MLH1 without concomitant loss of PMS2, combined losses (e.g., loss of MLH1/PMS2 and isolated loss of MSH6) or even complete loss of both MSH2/MSH6 and MLH1/PMS2 dimers).

Special attention should be given to tumors treated with chemotherapy and radiotherapy, which may present abnormal MSH6 expression unrelated to MSH6 mutation (exclusively nucleolar staining, nuclear staining in the tumor of weaker intensity than in the control, or heterogeneous staining). In these cases, MMR status evaluation should be confirmed in the pre-treatment biopsy (Bao et al. 2010).

Cytoplasmic expression of MMR proteins can also be seen, mainly due to over-fixation. However, cytoplasmic expression of MSH2 has been described in CRC patients with suspected Lynch syndrome, due to MSH2/EPCAM fusion (Sekine et al. 2017; Dong et al. 2021).

The pathologist may also encounter tumors that present areas with staining of all four MMR proteins and areas where the absence of expression of one or more proteins is observed (Joost et al. 2014). This staining heterogeneity has been described as intraglandular (in sections of the same gland), clonal (in entire glands or groups of glands), or compartmental (in different tumor compartments or different blocks tested) (Fig. 2). Although rare, pathologists should be aware of this phenomenon to avoid interpretation errors. When evaluated by molecular testing, most of these tumors are classified as MSI. However, some cases may be MSS by molecular testing due to partial functioning of the repair system (when isolated loss of MSH6 is partially compensated by MSH3, which forms an MSH2/MSH3 heterodimer). Cases with multifocal heterogeneous loss of MSH2/MSH6, MLH1/PMS2, or PMS2 may also be MSS by molecular testing (Guyot D'Asnières De Salins et al. 2021). In tumors where a phenotype change is observed (for example, in a tumor with well-differentiated and poorly differentiated areas or a tumor exhibiting areas with usual tubular and mucinous phenotypes), it is recommended to search the areas with different phenotypes to identify cases that may present compartmental heterogeneity.

As outlined above, several scenarios can complicate the analysis of MMR status by IHC. The latest version of the CAP guideline for biomarkers in CRC provides instructions on how to assess DNA repair system proficiency by IHC (College of American Pathologists 2021). This guideline emphasizes the importance of the presence of positive internal control for a valid reaction, as well as knowing the possibility of abnormal MSH6 staining in previously treated samples or MLH1/PMS2-deficient cases with mutations in the MSH6 gene. On the other hand, the CAP guideline states that "any positive reaction in the nuclei of tumor cells is considered as intact expression (normal), and it is common for intact staining to be somewhat patchy". This statement may raise questions about how to classify cases with heterogeneous staining (particularly in cases with heterogeneous intraglandular staining).

MSI testing by molecular methods

The evaluation of the DNA repair system by molecular methods (MSI testing) is usually performed using PCR-based methods and more recently by Next Generation Sequencing (NGS).

In PCR-based MSI testing, the presence of differences in microsatellite loci size involves the analysis of

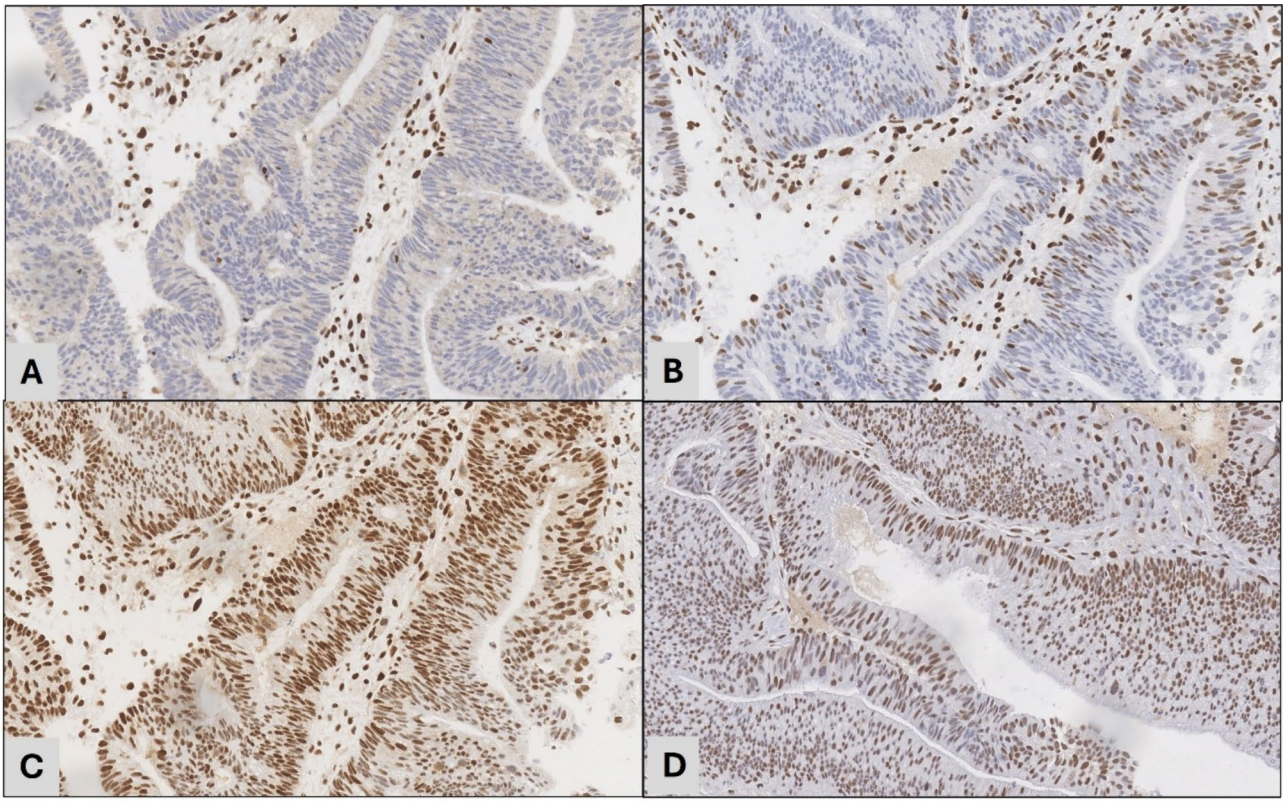

Fig. 2 Mismatch repair immunohistochemistry in colorectal cancer. MSH2 shows homogenous loss of staining (**a**). MSH6 shows a heterogeneous intraglandular loss pattern. Some neoplastic cells show retained nuclear staining while others show loss of staining within glandular formations (**b**). MLH1 and PMS2 show retained expression (**c**, **d**)

repetitive sequences of mononucleotides or a combination of mononucleotides and dinucleotides. Panels composed exclusively of mononucleotides are preferred for their higher sensitivity and specificity. There are several in vitro diagnostic tests currently available for evaluating MSI status. The Ydilla MSI assay (Ukkola et al. 2021) automatically evaluates DNA extracted from FFPE-embedded tumors, without the need for non-tumoral control inclusion, using a panel of 7 mononucleotide markers (ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, SULF2). A tumor is considered unstable (MSI) if alterations are observed in 2 or more loci. The validity of the test depends on a percentage of viable tumor cells greater than or equal to 20% and at least 5 markers considered valid.

MSI testing can also be performed using Next Generation Sequencing (NGS) techniques, with the advantage of obtaining additional information that can be used in therapeutic decision-making, such as determining Tumor Mutational Burden (TMB), *KRAS* status, and *HER2* status. This technique is still limited in Brazil, being used only in specialized centers. In addition, international guidelines recommend that MSI testing by NGS should be adopted only after its sensitivity and specificity have been compared with the use of MMR IHC or MSI testing by PCR (Luchini et al. 2019).

Table 1 Strategy for dealing with mismatch repair protein immunohistochemistry abnormal/uncommon results

Result	Strategy
Absence of staining in tumor cells and in the internal control.	Repeat the test. Use another block. If the result persists, consider MSI testing
Weaker tumor staining than the internal control	Repeat the test. If the result persists, consider it an equivocal/inconclusive result and consider MSI testing
Anomalous staining in post-neoadjuvant tumor	Perform the test on the pre-treatment biopsy. If pre-treatment biopsy is not available, consider MSI testing
Cytoplasmic staining (in the absence of nuclear staining)	Repeat the test. Use another block. If the result persists, consider it as loss of the protein being evaluated (dMMR)
Loss of all four MMR proteins	Check the internal controls. If the quality of the reaction is adequate, report as dMMR. Consider MSI testing
Loss of 2 proteins (no heterodimer)	Check the internal controls. If the quality of the reaction is adequate, report as dMMR. Consider MSI testing
Loss of 3 proteins	Check the internal controls. If the quality of the reaction is adequate, report as dMMR. Consider MSI testing
Heterogeneous staining of one or more proteins	Check for fixation artifact, particularly in surgical specimens. If the reaction quality is adequate, report as dMMR. Comment on the presence of heterogeneous/anomalous staining. Consider MSI testing

dMMR: mismatch repair deficient; MSI: microsatellite instability. Adapted from Chen and Frankel 2019; Bartley et al. 2022 and Vikas et al. 2023

Discordance in MMR/MSI testing: MMR IHC and MSI molecular testing should be friends, not foes

Discordance between MMR immunohistochemistry and molecular MSI testing occurs in up to approximately 10% of CRC cases, due to pre-analytical and/or biological factors (see above) (Bartley et al. 2012; Guyot D'Asnières De Salins et al. 2021).

While some authors advocate dual testing by MMR IHC and MSI molecular testing to eliminate the chance of not detecting dMMR cases (Dedeurwaerdere et al. 2021; Guyot D'Asnières De Salins et al. 2021), this approach has the disadvantage of consuming valuable material that may be needed in the future for other tests (particularly in needle biopsies of metastatic tumors). Moreover, the high cost of molecular tests in Brazil prevents their widespread adoption by the public healthcare system. Such tests are also not covered by private health insurance plans and must be paid for by the patient. Therefore, it is preferable to adopt MMR IHC as the initial test, reserving the assessment of MSI by molecular methods for cases where IHC results are inconclusive or equivocal (Luchini et al. 2019). Molecular testing is also recommended for cases with isolated losses of MSH6 or PMS2 due to suspected Lynch syndrome or in rare pMMR cases where there is a strong clinical suspicion of Lynch syndrome association. A cost-effective strategy to address different situations that pathologists may face, using MMR IHC as the initial test and reserving the use of MSI molecular testing for challenging cases, is summarized in Table 1.

RAS/RAF testing

Importance for clinical decision making

Around 50% of patients with CCR have a mutation in the RAS pathway. In general, every metastatic CRC will receive first-line standard chemotherapy combinations based on fluoropyrimidine and platinum (FOLFOX or CAPOX) or topoisomerase inhibitor (FOLFIRI), associated with a monoclonal antibody anti-VEGF or anti-EGFR. Both anti-VEGF and anti-EGFR drugs are well-accepted, intravenous drugs with similar efficacy when dealing with left side CRC, but with different side effects and treatment tolerability. One of the main factors that will guide patient treatment is the presence or absence of mutations in the RAS/RAF pathway.

Since the early 2000s, with the publication of BOND trial (Cunningham et al. 2004), it was known that adding an anti-EGFR antibody (in this case, Cetuximab) to CRC treatment in advanced and refractory lines could be a good salvage strategy and lead to a progression-free survival gain. However, some studies seemed to show that

treatment efficacy could be altered according to the presence of KRAS mutations in tumors (Lièvre et al. 2006). The randomized trial OPUS then proved that the efficacy of cetuximab combined with chemotherapy regimens occurs only in patients without KRAS mutations, a fact that was also later demonstrated for BRAF mutations (Yuan et al. 2013).

Thus, most patients with metastatic CRC and a known mutation in the RAS/RAF pathway will receive first-line chemotherapy associated with an anti-VEGF antibody, as there is no benefit in adding an anti-EGFR drug in this population.

Finally, for patients with BRAF mutations, targeted therapy is approved in later lines of treatment. The BEACON CRC trial (Tabernero et al. 2021) showed that for the population already refractory to chemotherapy and with BRAF V600E mutation, the use of encorafenib (tyrosine kinase inhibitor) associated with cetuximab brought overall survival gain compared to placebo (9 months vs. 6 months).

The importance of the pathologist for a reliable KRAS testing

It's important to highlight that mutations in the RAS/RAF family molecules cannot be reliably detected through conventional immunohistochemistry. Therefore, identification requires molecular pathology methods.

The pathologist's role in evaluating mutations in the RAS/RAF pathway involves different stages:

Selection of the ideal sample

In the process of selecting the ideal sample, both the primary tumor and metastatic lesions can be used since several studies have shown that the concordance rate of mutational status between them exceeds 90% (Artale et al. 2008; Baas et al. 2011). However, specimens resected after chemotherapy or radiotherapy should be avoided if the sample cellularity is low. Pre-treatment samples should be favored in this scenario (Boissière-Michot et al. 2012).

Sample qualification

This stage aims to ensure that the selected material has sufficient tumor representation for performing the molecular test of the RAS/RAF pathway. The estimation of the tumor cell fraction (TCF) is usually done by visually assessing the percentage of neoplastic cells relative to non-neoplastic cells in a given area. Studies have shown that this method of analysis has high interobserver variability (Smits et al. 2014; Mikubo et al. 2020), resulting in efforts to improve TCF assessment and minimize failures in routine tests (with incorrect or inconclusive results), or the incorrect exclusion of samples that would be suitable for testing.

While it is expected that with the more widespread use of Digital Pathology and Artificial Intelligence pathologists will be able to rely on automated methods to estimate the TCF (Frei et al. 2023; Sakamoto et al. 2022; L'Imperio et al. 2024), visual estimation currently remains the technique adopted in most laboratories.

To improve visual estimation, a recent consensus (Dufraing et al. 2019) recommended that:

1) TCF assessment be performed by pathologists. In laboratories where biologists/technicians estimate the TCF after specific training, a pathologist must be available for feedback.

2) The estimate should be made in the area with the highest density of neoplastic cells and the lowest density of other cell types (notably inflammatory cells, but also desmoplastic stroma, adipose tissue, or muscle tissue). Areas with extensive necrosis, mucus, or ulceration should be avoided.

3) Manual counting of individual cells should be avoided in daily routine due to impracticality. The percentage estimate should be rounded to the nearest 10%. (Fig. 3).

4) In samples requiring macrodissection, the pathologist should mark on the selected HE slide the area considered for TCF assessment (Fig. 3). Additionally, the selected area will be used as a reference when macrodissecting the corresponding sample on the unstained slide used in the molecular test.

Choice of test for RAS/RAF pathway status analysis

Different methods for detecting RAS/RAF mutations in CRC are currently available, such as RT-PCR, NGS, Sanger sequencing, or pyrosequencing. More than one method may be available in different laboratories. Pathologists involved in the sample evaluation flow for testing RAS/RAF mutational status should be aware of the tests used in their laboratory, in order to assess if a specific sample is suitable for the limit of detection (LoD) of the molecular test that will be used in a specific case.

HER-2 status

Importance for treatment decision making

HER-2 is an oncogene responsible for encoding one of the four proteins in the HER family, a group of transmembrane proteins whose activation via dimerization triggers intracellular signal transduction pathways, inducing cell proliferation and migration (Hayes 2019). Approximately 3 to 5% of CRC cases exhibit HER2 overexpression, which holds significant prognostic implications in clinical practice. Similar to breast cancer, HER2 protein overexpression in colorectal cancer (CRC) predominantly correlates with HER2 gene amplification. Notably, a significant proportion of HER-2 positive CRC cases lack mutations in

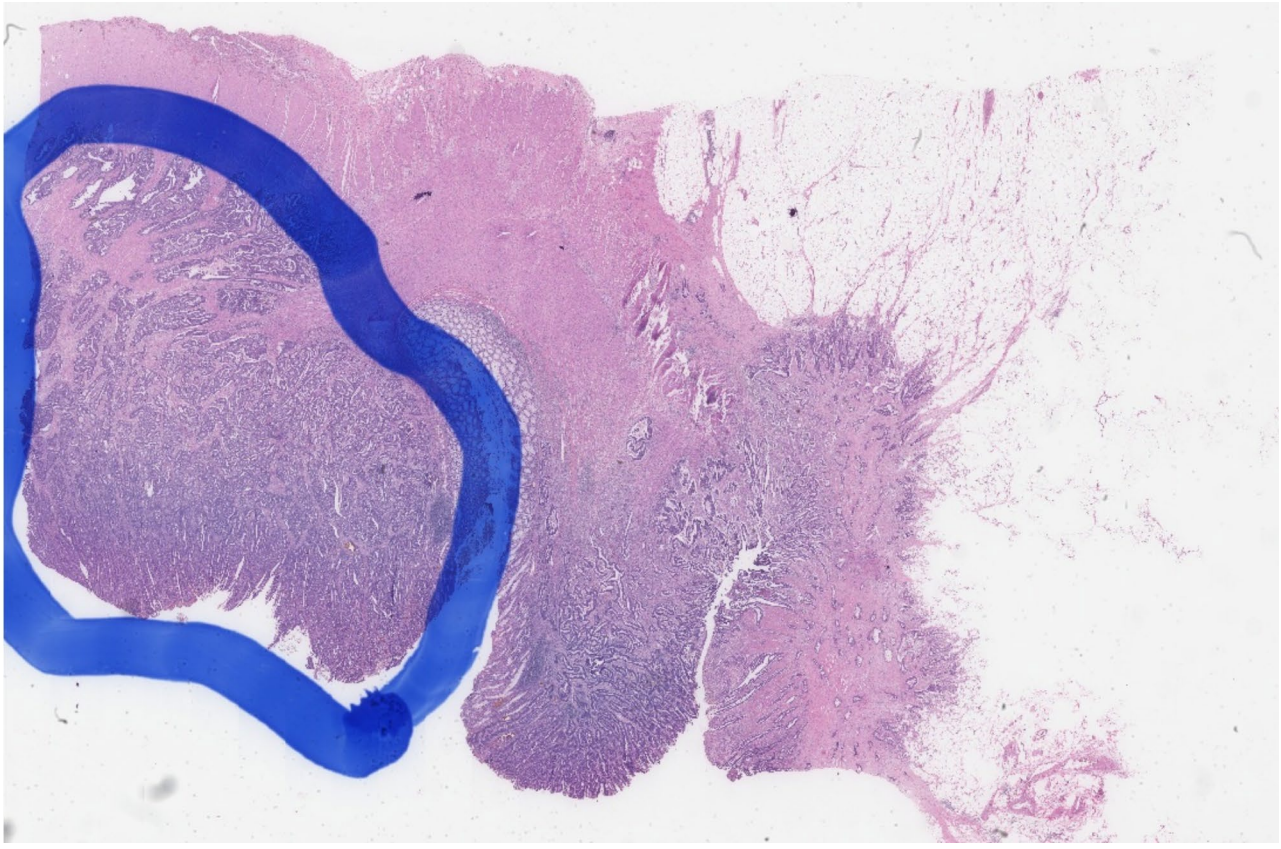

Fig. 3 Hematoxilin and Eosin section of colorectal adenocarcinoma with zone delineated for microdissection prior to molecular KRAS testing with 70% neoplastic cells

the RAS/RAF pathway, suggesting a key role for HER2 as a driver in these tumors' pathogenesis (Ross et al. 2018).

Assessment of HER2 status in CRC can impact treatment decisions in advanced stages. The HERACLES study was a phase 2 trial that administered intravenous trastuzumab combined with oral lapatinib to standard treatment in refractory CRC patients, evaluating the anticancer efficacy of these agents in HER2-overexpressing tumors (Sartore-Bianchi et al. 2016). Approximately 30% of treated patients exhibited partial response, and 40% achieved disease stabilization.

The MyPathway study was pivotal in demonstrating the benefits of targeting the HER2 pathway in CRC (Meric-Bernstam et al. 2019). Patients with HER2-positive status, as determined by immunohistochemistry (IHC), received combination therapy with trastuzumab and pertuzumab in third-line or later settings. This treatment resulted in a 32% objective response rate and a median overall survival of approximately 11 months.

The landscape for HER2-positive CRC has significantly evolved with the approval of the antibody-drug conjugate Trastuzumab Deruxtecan (T-DXd). This molecule has shown promise in treating advanced disease across various histologies with HER2 overexpression/amplification, being approved as an agnostic therapy in later lines of treatment. In CRC, the DESTINY-CRC01 study demonstrated the benefit of T-DXd in patients who progressed after 2 or more lines of treatment, with confirmed HER2 3+by IHC or 2+IHC with amplification confirmed by in situ hybridization (Yoshino et al. 2023). Notably, in CRC, unlike other histologies, T-DXd does not appear to be effective for tumors classified as HER-2 low (1+or 2+with no gene amplification).

HER2 IHC/ISH reporting in CRC – still an unresolved issue

Pathologists may be tasked to evaluate HER2 status in CRC similarly to how it is routinely done in gastroesophageal carcinoma and breast carcinoma, for which specific guidelines have been developed and widely adopted. Guidelines for breast and gastric cancers use different criteria for positive/negative characterization. However, there is no current universally accepted formal guideline for HER2 assessment in CRC and each clinical trial has used its own.

The HERACLES study established specific criteria (Heracles Diagnostic Criteria) for patient selection (Valtorta et al. 2015). A case was considered positive (IHC score 3+) if≥50% of neoplastic cells exhibited intense,

circumferential, lateral, or basolateral staining. In this scenario, confirmation by in situ hybridization was unnecessary. Conversely, cases with an IHC score of 3+in >10% or <50% of neoplastic cells, though considered positive, required confirmatory retesting by immunohistochemistry. Cases with a score of 2+ (moderate circumferential, lateral, or basolateral staining in ≥50% of neoplastic cells) were deemed "equivocal," mandating retesting followed by in situ hybridization amplification testing. The highly restrictive nature of the Heracles Diagnostic Criteria is further emphasized by the fact that patients with a score of 3+in <10% of neoplastic cells or 2+in <50% of neoplastic cells were deemed negative and ineligible for the HERACLES study. It should be noted that the evaluation of HER2 in CRC using the Heracles Diagnostic Criteria was recommended by the NCCN in its latest CRC treatment guideline (National Comprehensive Cancer Network 2024). However, the feasibility of transferring a protocol adopted in a clinical trial with central laboratory review was not addressed. If laboratories are to follow this protocol without adaptations, cases with scores of 2+or 3+should be retested by immunohistochemistry before proceeding to in situ hybridization testing (for cases with 2+≥50% of neoplastic cells or 3+>10% or <50% of neoplastic cells). In Brazil, as in many countries, repeating HER2 IHC testing to confirm the findings of the initial examination is not viable or reasonable, whether due to patient budget constraints, noncompliance with public healthcare system guidelines, or lack of approval by private healthcare systems. Therefore, the question remains open as to whether the HERACLES protocol can be adapted for daily practice by excluding the step of HER2 IHC retesting and referring cases with 2+≥50% of neoplastic cells or 3+>10% or <50% of neoplastic cells for in situ hybridization testing.

The MyPathway study used criteria adopted for gastroesophageal adenocarcinomas (CAP/ASCP/ASCO gastroesophageal adenocarcinoma HER2 guideline) (Bartley et al. 2016). This criterion is less restrictive, considering cases with 3+IHC score in ≥10% of neoplastic cells as positive, in addition to cases with 2+IHC score in ≥10% of neoplastic cells and HER2 amplification by in situ hybridization testing. The DESTINY-CRC01 study utilized the selection criterion of IHC3+or IHC2+with HER2 amplification by in situ hybridization. Although this study does not provide further information regarding the criteria used, it appears similar to that adopted in the MyPathway study.

A comparison of the different criteria is presented in Table 2. A case with a 2+IHC score that can have different interpretations depending on the criteria adopted is depicted in Fig. 4. Harmonization efforts are underway (Fujii et al. 2020), but until a consensus is reached, the latest version of the CAP guideline for biomarkers in CRC (College of American Pathologists 2021) recommends that pathologists report the staining intensity, the percentage of tumor cells with specific membrane staining, the test result and the score that was used in the analysis. A structured template for HER2 IHC reporting is presented in Table 3.

Likewise, different criteria may be adopted for cases referred for ISH testing. The MyPathway study used criteria from CAP/ASCP/ASCO, where amplification is defined by a HER2:CEP17 ratio≥2.0 in 10% of neoplastic cells or >6, even in the absence of HER2:CEP17 ratio≥2.0, in cases where co-amplification of HER2 and

Table 2 HER2 immunohistochemistry results, interpretation, and consequences according to CAP/ASCP/ASCO gastrointestinal carcinoma, HERACLES and MyPathway criteria

HER2 IHC Result	CAP/ASCP/ASCO gastrointestinal adenocarcinoma criteria interpetation and consequencess	HERACLES Diagnostic Criteria interpretation and consequences
No reactivity of membranous reactivity in < 10% of tumor cells	**Score 0 - Negative**. No further testing. Patient not eligible for therapy	**Score 0 - Negative**. No further testing. Patient not eligible for therapy
Faint/barely perceptible reactivity in ≥ 10% of tumor cells	**Score 1+ - Negative** . No further testing. Patient not eligible for therapy	**Score 1+ - Negative**. No further testing. Patient not eligible for therapy
Weak to moderate complete, basolateral or lateral membranous staining in ≥ 10% but < 50% of tumor cells	**|Score 2+ - Equivocal**. Perform ISH testing.	**Score 2+ - Negative**. No further testing. Patient not eligible for therapy
Weak to moderate complete, basolateral or lateral membranous staining in ≥ 50% of tumor cells	**Score 2+ - Equivocal**. Perform ISH testing	**Score 2+ - Equivocal**. Mandatory IHQ re-test required. If ≥ 50% cellularity confirmed, perform ISH testing. Patient eligible for therapy if ISH positive
Strong complete, basolateral or lateral membranous staining in ≥ 10% but < 50% of tumor cells	**Score 3+ - Positive**. No further testing. Patient eligible for therapy	**Score 3+ - Positive**. Mandatory IHQ re-test required. If > 10% cellularity confirmed, perform ISH testing. Patient eligible for therapy if ISH positive
Strong complete, basolateral or lateral membranous staining in ≥ 50% of tumor cells	**Score 3+ - Positive**. No further testing. Patient eligible for therapy	**Score 3+ - Positive**. No further testing. Patient eligible for therapy

IHC immunohistochemistry; ISH: in situ hybridization. Adapted from Bellizzi 2020, Valtorta et al. 2015

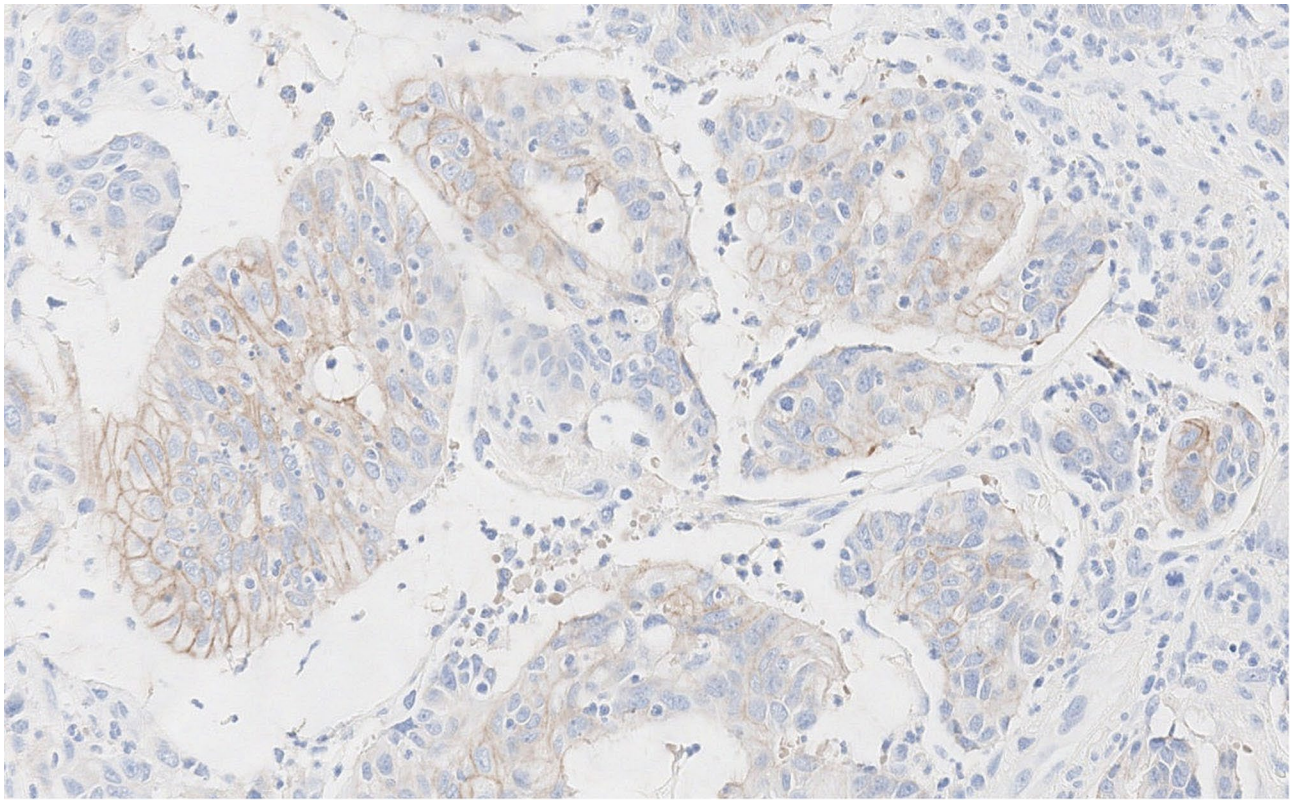

Fig. 4 HER2 immunohistochemistry in a colorectal adenocarcinoma. This tumor showed a 2 + score (moderate circumferential, lateral, or basolateral staining) in 30% of neoplastic cells. According to the "CAP/ASCP/ASCO gastroesophageal adenocarcinoma HER2 guideline", it should be classified as "equivocal" and sent to in situ hybridization testing for assessment of *HER2* amplification status. According to the "Heracles Diagnostic Criteria", it should be classified as negative, with no further action taken. Original magnification 200x

Table 3 Proposed template for HER2 IHC reporting (according to CAP 2021 guidelines recommendations for reporting biomarkers in CRC)

Items to be reported	Variables
Staining Intensity	[ ] 0 (none) [ ] 1 (faint or barely perceptible) [ ] 2 (weak to moderate) [ ] 3 (strong)
Percentage of tumor cells with specific membranous staining (i.e., complete, basolateral or lateral membrane)	[ ] Less than 10% [ ] 10–49% [ ] Greater than or equal to 50% [ ] Specify percentage: %
HER-2 IHC Result	[ ] Negative [ ] Equivocal [ ] Positive
Which score was used in the report	[ ] CAP/ASCP/ASCO HER2 Gastroesophageal Adenocarcinoma (aka Ventana) [ ] HERACLES Diagnostic Criteria [ ] HER2 Breast Cancer 2018 score [ ] Others (specify)

CAP: College of American Pathologists; CRC: colorectal cancer; IHC: immunohistochemistry

CEP17 is observed. The HERACLES study criteria, on the other hand, considered cases amplified only if they exhibited a HER2:CEP17 ratio ≥ 2.0 in 10% of neoplastic cells. Therefore, the latest version of the CAP guideline for biomarkers in CRC also recommends that pathologists report which criteria were used in the ISH analysis for the cases submitted to ISH testing.

ctDNA monitoring of post-surgery-CRC patients

Importance for clinical decision making

The next predictive factor expected to be integrated into CRC treatment is the monitoring of circulating tumor DNA (ctDNA). Malignant tumors typically release DNA into circulation, and the detection of ctDNA is now recognized as a highly sensitive method for monitoring malignant cell presence, even when tumors are undetectable by standard tests. Studies have shown that CRC patients have higher levels of ctDNA in their bloodstream compared to other solid tumors (Nakamura et al. 2020; Vymetalkova et al. 2018). Additionally, ctDNA levels vary according to the stage of the disease, with higher levels of detection seen in stage IV patiens (Bettegowda et al. 2014; Tie et al. 2015). This feature of CRC tumors (i.e. shedding ctDNA in the bloodstream) and the

advancement of ctDNA detection techniques using blood samples has led to intense investigations into the role of this biomarker in monitoring and prognosis of CRC patients, as well as in the selection of more effective and less adverse treatment plans.

A complete revision of the potential diverse roles of ctDNA in CRC management is beyond the scope of this paper and the reader is referred to the literature for further details (Malla et al. 2022; Krell et al. 2023). The predictive role of this biomarker has been investigated mainly in stage II and III CRC, where approximately 50% of patients are cured without the need for adjuvant therapy. The randomized phase II DYNAMIC trial demonstrated that adjuvant chemotherapy could be safely omitted, without compromising disease-free survival rates, for stage II CRC (T3 or T4, N0, M0) with a negative ctDNA test seven weeks post-surgery (Tie et al. 2022).

In stage III CRC, a multicenter study followed 96 newly diagnosed patients with serial plasma samples collected after surgery and after adjuvant chemotherapy (Tie et al. 2019). Patients with detectable ctDNA had lower recurrence-free intervals (RFI) than those with undetectable ctDNA (30% *versus* 77% estimated 3-year RFI). This study indicates that stage III patients that are at high risk of recurrence after surgery and standard chemotherapy may benefit from extended adjuvant treatment or additional therapeutic options.

The results of these and other ongoing interventional studies (Malla et al. 2022) will help in the establishment of the utility of ctDNA in better selecting treatment options for CRC patients. Thus, with the incorporation of molecular pathology methods into clinical practice, the indication for adjuvant chemotherapy in stage II-III CRC may be altered.

The importance of traditional pathology diagnosis for ctDNA testing

Although ctDNA testing is performed on blood samples, pathologists also have a role in this scenario. Pathological staging of the surgical specimen determines the patient's clinical stage and potential eligibility for this test. Therefore, pathologists must be aware of potential pitfalls that may lead to staging errors:

- pT2 vs. pT3 tumor: pathologists often encounter tumors with invasion extending to the interface between the muscularis propria and the subserosa, making it challenging to correctly determine whether a case should be classified as pT2 or pT3. In this scenario, level sampling and immunohistochemical evaluation with antibodies to highlight the muscularis propria (desmin, smooth-muscle actin, caldesmon) are indicated.
- Inadequate or borderline number of available lymph nodes for analysis: twelve lymph nodes are the minimum acceptable number of regional lymph nodes for pN staging evaluation. However, given the importance of determining the absence of lymph node metastases (pN0) to classify a patient with a pT3 or pT4 tumor as Stage II, a thorough examination of the specimen and submission of all identified lymph nodes for processing is mandatory to increase the likelihood of identifying a positive lymph node (Lykke et al. 2015).
- Direct invasion of a lymph node by the tumor front: in this scenario, pathologists may be unsure whether to designate the lymph node as positive or negative. The UICC recommends that, even if invasion occurs by contiguity, the lymph node should be designated as positive (Wittekind et al. 2019).
- Confirmation of the presence of tumor deposits: in the absence of regional lymph node involvement, the presence of tumor deposits classifies a tumor as pN1c. Therefore, it is important to recognize the main mimickers, which include infiltration around nerves and the presence of tumor thrombi filling the lumen of large extramural venous vessels. In the latter, rounded tumor nodules without evidence of residual lymphoid tissue are often seen in the subserosa and pericolorectal adipose tissue (College of American Pathologists, 2023). The presence of an adjacent artery without a corresponding vein ("orphan artery phenomenon") allows the diagnosis of these nodules as extramural veins filled with tumor thrombi (Odze and Goldblum 2023).

Conclusion

The incidence of CRC has been increasing worldwide, and Brazil is no exception. In this context, the rise in cases of CRC among people younger than 50 years poses additional challenges to the prevention, diagnosis, and treatment of this disease, which can cause severe harm.

Significant progress has been made in recent years in the development of targeted therapies and the improvement of predictive biomarkers to assist in the treatment planning for CRC. Currently, the therapeutic management of CRC involves routine testing to assess DNA repair proficiency (which can alter therapeutic planning in patients with localized early-stage disease), as well as the mutational status of RAS and the analysis of HER2 status (used in therapeutic decision-making for patients with advanced CRC).

In Brazil, the evaluation of DNA repair proteins by MMR IHC is already routinely performed in pathology laboratories. The interpretation of MMR immunohistochemistry is straightforward in most cases

(showing either maintenance of nuclear staining or complete absence of staining in neoplastic cells). However, anomalous staining patterns can be difficult to interpret. Pathologists should recognize staining alterations that may occur in cases with inadequate fixation. They should also be aware of the heterogeneous staining patterns that have been described and may arise in routine diagnostics as more cases are evaluated, in order to assess, in consultation with the oncologist, the relevance of performing additional MSI testing in such cases.

Similarly, the assessment of HER2 immunoexpression can also be conducted in most laboratories, although pathologists need to familiarize themselves with the scoring systems and reach an agreement with the oncology team regarding which scoring system should be adopted, while the issue of harmonization remains open. Although the implementation of ISH for HER2 status assessment is available in fewer laboratories, the same question of which scoring system will be used should be a subject of discussion between the pathologist and the oncologist, keeping in mind that the system to be used should be the same as that adopted for previous immunohistochemical analysis.

Predictive tests based on molecular assays (MSI, RAS, HER2) are usually restricted to large oncology centers. Nevertheless, gastrointestinal and general pathologists should be aware of these tests, as they play a central role in the clinical management of CRC patients, which requires accurate diagnosis and staging, assessment of sample adequacy for immunohistochemical and molecular testing, and proper interpretation of results. Additionally, the effective use of these biomarkers requires that pathologists and oncologists be aware of the factors that can influence the quality of a specific test and consequently the reliability of the results.

Recently, the use of liquid biopsy for ctDNA assessment in blood samples as a predictive factor has gained relevance, following recent findings from clinical studies investigating its role in stage II-III CRC patients for the adoption of de-escalation or escalation therapeutic strategies. The potential importance of this biomarker in CRC management can still be confirmed by several ongoing trials examining its role in various diagnostic and treatment scenarios. Although this molecular test may not be part of the daily routine for a significant portion of pathologists, they should keep in mind the essential role that accurate pathological diagnosis and staging will play in the use of this biomarker for therapeutic decision-making.

Finally, the rapid advancements in predictive markers and treatment options witnessed over the past decades would not have been possible without the use of well-characterized cancer samples. Therefore, hospitals, research centers, and the medical team involved in the diagnosis and care of oncology patients should recognize this importance and direct efforts towards the preservation of oncological samples in biobanks (Annaratone et al. 2021). This practice will streamline future analyses and further progress in the field.

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