

PhD Programme in
Medicine and Molecular Oncology

**EVALUATION OF CTDNA AND OTHER CIRCULATING BIOMARKERS AS
MARKERS OF RESPONSE AND PROGNOSIS AFTER NEOADJUVANT
CHEMORADIOTHERAPY IN LOCALLY ADVANCED RECTAL CANCER**

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AS MARKERS OF RESPONSE AND PROGNOSIS TO NEOADJUVANT
CHEMORADIOTHERAPY IN LOCALLY ADVANCED RECTAL CANCER**

**AVALIAÇÃO DO CTDNA E OUTROS BIOMARCADORES
PLASMÁTICOS COMO MARCADORES DE RESPOSTA E
PROGNÓSTICO APÓS QUIMIOTERAPIA NEOADJUVANTE
NO CANCRO DO RETO LOCALMENTE AVANÇADO**

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

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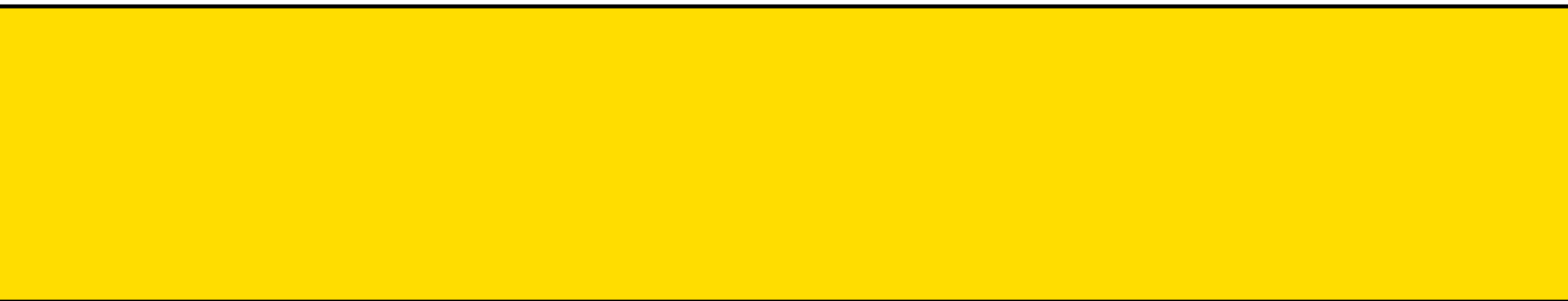
TABLE OF CONTENTS

ABBREVIATIONS	1
ABSTRACT	5
RESUMO	11
BACKGROUND.....	17
1. Epidemiology	19
2. Anatomy and Pathophysiology	20
3. Carcinogenesis and Progression	21
4. Diagnosis and Staging	24
4.1. Future Methods of Clinical Staging.....	26
5. Current Management of Patients with LARC.....	28
5.1. Surgery.....	28
5.2. Neoadjuvant Treatment.....	29
5.2.1. Radiotherapy.....	29
5.2.2. Chemotherapy.....	31
5.3. Tumor Pathological Response and Organ Preservation Strategies.....	31
5.3.1. Current Predictors of Response	33
5.4. Adjuvant Treatment.....	35
5.5. Novel Therapeutic Options for LARC.....	36
6. Survival and Follow-up	38
7. Novel Biomarkers for Prediction of Response and Prognosis	41
7.1. Tissue-Based Biomarkers	42
7.2. Blood-Based Biomarkers	46
THESIS OVERVIEW: AIMS AND METHODS.....	57
RESULTS	61
Article 1.....	63
Article 2.....	75
Article 3.....	91
Article 4.....	102
DISCUSSION	117
1. PN Index and PLR Play a Role in Predicting Response to nCRT in LARC Patients	121

2. NLR and PN index Can Predict Survival Outcomes in LARC, Adding Value to TNM Stage	123
3. ctDNA is a Thriving Predictive Tool for Assessing Response and Prognosis in LARC Patients	126
4. Prediction of response through imaging features could be complemented with ctDNA analysis	129
FUTURE PERSPECTIVES.....	133
CONCLUSION	137
BIBLIOGRAPHY	141
CURRICULUM VITAE	157

On the elaboration of the text of this thesis, we adopted North American medical English (**NAmE**) terminology because the published papers behind this thesis are written in the same language.

ABBREVIATIONS



ABBREVIATIONS

#	DRE digital rectal examination
5-FU 5-fluorouracil	
A	E
AGR albumin-globulin ratio	EGF epidermal growth factor
AJCC American Joint Committee on Cancer	EGFR epidermal growth factor receptor
APC adenomatous polyposis coli	EMT epithelial mesenchymal transition
ASCO American Society of Clinical Oncology	EMVI extramural vascular invasion
	EpCAM epithelial cellular adhesion molecule
B	ERUS endorectal ultrasound
Bcl2-bax B-cell lymphoma protein 2-associated X	ESMO European Society of Clinical Oncology
bp base pairs	exoDNA/RNA exosome-derived DNA or RNA
BRAF V-Raf murine sarcoma viral oncogene homolog B	
	F
C	FA score plasma fibrinogen and serum albumin
CA 19-9 carbohydrate antigen 19-9	FDA Food and Drug Administration
CAPOX capecitabine plus oxaliplatin	FOLFIRINOX 5-FU, leucovorin, oxaliplatin and irinotecan
CAR C-reactive protein-albumin ratio	FOLFOX 5-FU, leucovorin and oxaliplatin
cCR complete clinical response	FoxP3 forkhead box P3
CD cluster of differentiation	
CE conformité européenne	G
CEA carcinoembryonic antigen	mGPS modified Glasgow prognostic score
cfDNA circulating free DNA	Gy Grey
CIMP CpG island methylator phenotype	
CMS Consensus Molecular Subgroups	K
COX-2 cyclooxygenase 2	KRAS Kirsten rat sarcoma viral oncogene homolog
CRC ColoRectal Cancer	
CRCSC Colorectal Cancer Subtyping Consortium	I
CRIS CRC intrinsic subtypes	IWWD International Watch and Wait Database
CRM circumferential resection margin	IMRT intensity-modulated radiation therapy
CRP C-reactive protein	IORT intraoperative radiation therapy
CT computed tomography	IPI inflammation-related prognostic index
CTC circulating tumor cells	
ctDNA circulating tumor DNA	L
	LA lymphocyte count × albumin level
D	LARC locally advanced rectal cancer
DCC deleted in colorectal cancer	LCR lymphocyte-to-C-reactive protein ratio
DFS disease-free survival	LMR lymphocyte-to-monocyte ratio
dMMR deficient mismatch repair	LOH loss of heterozygosity
DNA deoxyribonucleic acid	M
dPCR digital polymerase chain reaction	MAF mutation allele frequency
	MAPK mitogen-activated protein kinases

ABBREVIATIONS

mCRC metastatic colorectal cancer
MDT multidisciplinary team
MERCURY MRI and Rectal Cancer European Equivalence trial
MiR micro-RNA
MLH mutL homolog
MSH mutS homolog
MMR mismatch repair
mRNA messenger RNA
MRD microscopic residual disease
mrEMVI MRI extramural venous invasion
MRI magnetic resonance imaging
mrTRG MRI TRG
MSI microsatellite instability
MSKCC Memorial Sloan Kettering Cancer Center

N

N × M neutrophil × monocyte
NCCN National Comprehensive Cancer Network
nCRT neoadjuvant chemoradiotherapy
NCT National Clinical Trial
NGS next-generation sequencing
NLR neutrophil-to-lymphocyte ratio
NRAS neuroblastoma Ras viral oncogene homolog

O

OPRA Organ Preservation in Rectal Adenocarcinoma trial
OS overall survival

P

pCR pathological complete response
PD-1 programmed cell death 1
PET positron emission tomography
PFS progression-free survival
PhD Philosophy Doctor
PI3K phosphoinositide 3-kinases
PIK3CA phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PLR platelet-to-lymphocyte ratio
PN index platelet neutrophil index
PNI prognostic nutritional index
PTEN phosphatase and tensin homolog

Q

qPCR quantitative polymerase chain reaction

R

RAF rapidly accelerated fibrosarcoma
RAPIDO Rectal cAncer and Preoperative Induction therapy followed by Dedicated Operation trial
RNA ribonucleic acid

S

SCNA somatic copy number alterations
SCRT short-course radiotherapy
SII systemic inflammation index
SMD2 S-adenosylmethionine decarboxylase proenzyme
SMAD4 mothers against decapentaplegic homolog4
SNP single nucleotide polymorphisms
SUV standardized uptake value

T

TGF-β (transforming growth factor beta)
TIL Tumor infiltrating lymphocytes
TMB Tumor mutational burden
TNM tumor-node-metastasis
TNT total neoadjuvant therapy
TP53 tumor protein p53
TRG Tumor regression grade
TS thymidylate synthase

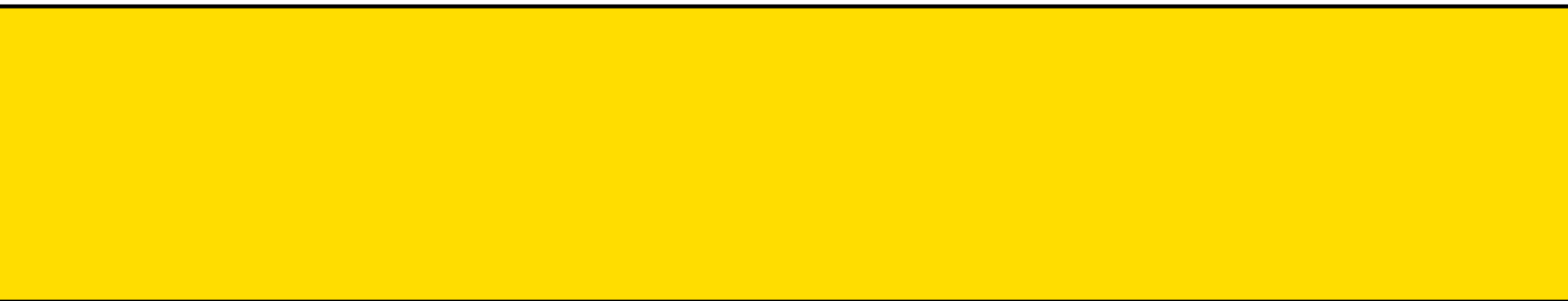
U

UICC Union for International Cancer Control

V

VAF variant allelic frequency
VEGF vascular endothelial factor

ABSTRACT



ABSTRACT

Background

Colorectal cancer is a large worldwide health problem, with a mortality of about 12%. Rectal cancer accounts for approximately 25-30% of all CRC. This work is focused primarily on patients with LARC, which is clinically defined as stage II/III.

For LARC patients, standard treatment is curative surgery and pre-operative radiotherapy, with or without concurrent chemotherapy. TNM staging system has been the standard to stage and guide treatment of these patients, however a high heterogeneity of outcomes inside the same TNM stage has been reported.

After neoadjuvant treatment, approximately 10-20% of patients reach pCR with a decreased likelihood of local recurrence and an improved survival. Organ-sparing strategies have been suggested for patients who achieve cCR to circumvent the complications of surgery. However, cCR and pCR appear to have poor concordance in patients with rectal cancer. Also, other strategies such as total neoadjuvant therapy have emerged with the intent of treating occult micrometastases in advance, as most of the patients recur with distant disease and the role of adjuvant therapy in this problem has been conflicting.

To stratify patients and provide personalized strategies, there is still a limited number of biomarkers. Currently, only imaging tumor response and post-treatment circulating CEA levels are clinically used.

A review of tissue- and blood-based biomarkers of growing interest in the literature was performed. The identification of these biomarkers in liquid biopsy could make them potential “real-time” disease biomarkers for disease monitoring. However, in LARC patients undergoing chemoradiotherapy, a variant or signature associated with treatment resistance or sensitivity is yet to be identified.

Aims

The search for more specific and readily available circulating biomarkers is a research field in expansion. This research project aimed to identify circulating markers that could be readily available in liquid biopsy and evaluate its efficacy in predicting response to nCRT and prognosis in LARC patients, such as hematological and biochemical parameters, and ctDNA.

Methods

In order to study the impact of baseline hematological and biochemical parameters in response to nCRT in LARC patients, a retrospective analysis of 191 patients at two Portuguese centers was performed. A logistic regression analysis intended to search for predictive markers of pathological complete and good response. To study the value of these markers in prognosis, a retrospective analysis of 245 patients was made in two centers. A Cox regression analysis was performed to search for predictors of prognosis. Survival outcomes were compared by Kaplan-Meier method to define subgroups of prognosis.

Then, to understand the impact of ctDNA in prediction of response and prognosis of LARC patients after treatment, a systematic review of the studies available in literature was performed and 21 publications were retrieved, with a total of 1499 patients studied. Then, a prospective study including 24 plasma samples of 18 patients of 3 Iberian centers intended to contribute with original data on the role of ctDNA in response to nCRT and prognosis of LARC patients.

Results

In the first published article included in this thesis, a high platelet-neutrophil index and clinical tumor stage >2 were predictive of poor response and none of the other analyzed biomarkers predicted pCR in this study. These included NLR, PLR, LMR, SII and prognostic nutritional index. In article 2, post-treatment PLR predicted

pCR. NLR at two timepoints of the treatment significantly predicted overall survival, whereas the post-nCRT PN index significantly predicted disease-free survival. In pathological stage II, the PN index predicted patients with a higher risk of disease-free survival. Article 3 has shown that data about the usefulness of liquid biopsy in this setting is still inconclusive. However, baseline higher levels of longer fragments of cell-free DNA and integrity index, tumor-specific mutations and certain methylated genes could predict non-responders. Also, undetectable baseline ctDNA and decrease of common rectal cancer mutations throughout treatment (dynamic monitoring) were predictive factors of pathological complete response. Furthermore, the continuous detection of ctDNA in different timepoints of treatment (minimal residual disease) was consistently associated with worse prognosis. In article 4, although the results should be read carefully due to small sample size, we suggest that baseline ctDNA combined with mrEMVI could potentially help predict response and baseline ctDNA number of mutations might allow the discrimination of groups with different DFS.

Conclusions

This thesis has contributed to the improved understanding of the value of circulating biomarkers in the prediction of response to nCRT and prognosis of LARC patients.

The benefits of identifying patients who would not respond to therapy could improve quality of life by avoiding unnecessary exposure to cytotoxic agents. Also, a more aggressive approach to treatment could be more appropriate in poor responders. Furthermore, the emergence of organ-sparing strategies would likewise benefit from accurate and reliable markers.

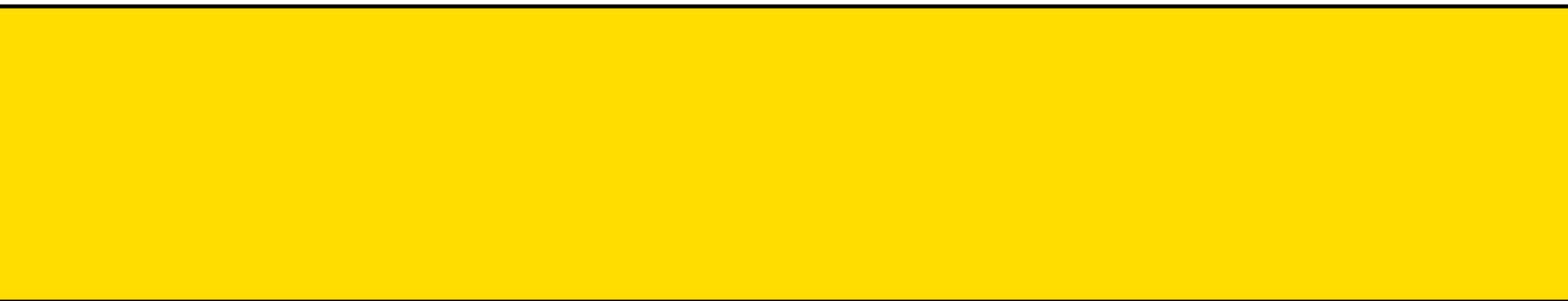
The formulation of scores and nomograms using hematological and biochemical markers already in use in clinical practice, could aid in subgrouping high and low-risk patients inside TNM stage. The ideal methods and timings for liquid biopsy require validation, but for the time being, serial analysis of specific mutations in ctDNA at the most opportune timings throughout treatment, added to

ABSTRACT

the available imaging methods, appears to be the most accurate way to make clinical decisions in the future.

However, the prediction of outcomes in LARC may not be possible using tumor-specific variants alone. The emergence of novel subtyping systems warn that variable expression profiles might have to be studied in order to predict response and prognosis, and that specific variants alone might not be significant enough. The inclusion of the markers and mutations studied in this thesis in existing staging systems, might help stratify patients' risk and ultimately optimize the treatment, survival and quality of life of LARC patients.

RESUMO



RESUMO

Introdução

O cancro colo-retal continua a ser um grande problema de saúde em todo o mundo, com uma mortalidade de 12%. O cancro do reto representa 25-30% de todos os cancros colo-retais. Este trabalho focou-se principalmente nos doentes com cancro de reto localmente avançado, que se define clinicamente por estádios II/III.

Para estes doentes, o tratamento habitual passa por cirurgia curativa e radioterapia pré-operatória, com ou sem quimioterapia concomitante. O sistema de estadiamento TNM está atualmente estabelecido para estadiar e guiar o tratamento destes doentes, apesar da grande heterogeneidade de prognóstico existente dentro de cada estágio do TNM.

Após o tratamento neoadjuvante, cerca de 10-20% dos doentes apresenta uma resposta patológica completa, associada a diminuição da recorrência local e melhoria da sobrevivência. Foram assim sugeridas estratégias de preservação de órgão para os doentes que alcançam uma resposta clínica completa ao tratamento, para tentar evitar as complicações da cirurgia. Contudo, as respostas clínica e patológica são pouco concordantes em doentes com cancro do reto. Para além disso, também têm aparecido outras estratégias como o tratamento neoadjuvante total, com o intuito de tratar micrometástases ocultas logo no início do tratamento, já que a maior parte dos doentes recidiva à distância e o papel da quimioterapia adjuvante neste problema é controverso.

Para estratificar os doentes e proporcionar estratégias personalizadas, ainda há um número limitado de biomarcadores. Atualmente, a resposta tumoral imagiológica e os níveis de CEA após o tratamento são os únicos fatores usados na prática clínica.

Nesta tese, foi efetuada uma revisão dos biomarcadores tecidulares e plasmáticos de interesse crescente na literatura. A identificação destes marcadores em biópsia líquida poderá torná-los biomarcadores “em tempo real” de monitorização da doença. Contudo, nos doentes com cancro do reto localmente

RESUMO

avanzado submetidos a quimiorradioterapia, ainda estão por identificar variantes ou assinaturas genéticas associadas a sensibilidade ao tratamento e prognóstico.

Objetivos

A procura de biomarcadores da circulação mais específicos e disponíveis na clínica é um campo de investigação em expansão. Este projeto de investigação pretendeu identificar marcadores plasmáticos disponíveis em biopsia líquida e avaliar a eficácia dos mesmos na previsão de resposta ao tratamento neoadjuvante e prognóstico nos doentes com cancro do reto localmente avanzado, como parâmetros hematológicos e bioquímicos, e o ctDNA.

Métodos

Para estudar o impacto dos parâmetros hematológicos e bioquímicos pré-tratamento na resposta dos doentes com cancro do reto localmente avanzado ao tratamento neoadjuvante, foi efetuada uma análise retrospectiva de 191 doente de dois centros portugueses. A regressão logística tentou identificar marcadores preditivos de resposta patológica completa e de boa resposta. Para estudar o valor destes marcadores no prognóstico, foi elaborada uma análise retrospectiva de 245 doentes em dois centros. A análise de regressão Cox foi feita para identificar preditores de prognóstico. Os resultados de sobrevivência foram comparados pelo método de Kaplan-Meier para definir subgrupos de prognóstico.

Em seguida, para compreender o impacto do ctDNA na previsão da resposta e de prognóstico nos doentes com carcinoma do reto localmente avanzado após tratamento, procedeu-se à realização de uma revisão sistemática dos estudos disponíveis na literatura, encontrando-se 21 publicações, com um total de 1499 doentes estudados. Depois, foi efetuado um estudo prospetivo de que incluiu 24 amostras plasmáticas de 18 doentes de 3 centros ibéricos para contribuir com novos dados em relação ao papel do ctDNA na resposta à quimiorradioterapia

neoadjuvante e no prognóstico dos doentes com carcinoma do reto localmente avançado.

Resultados

No primeiro artigo publicado incluído nesta tese, um índice plaqueta-neutrófilo alto e estágio tumoral clínico > 2 foram fatores preditivos de má resposta e nenhum dos outros fatores conseguiu prever resposta patológica completa neste estudo. Os fatores incluíram o NLR, PLR, LMR, SII e índice nutricional prognóstico. No artigo 2, o rácio plaqueta-linfócito pós-tratamento previu resposta patológica completa. O rácio neutrófilo-linfócito em dois pontos do tratamento conseguiu prever significativamente a sobrevivência global, enquanto o índice plaqueta-neutrófilo pós-tratamento permitiu prever significativamente a sobrevivência livre de doença. No estadio patológico II, este índice também permitiu a definição de subgrupos de mais alto risco de sobrevivência livre de doença. O artigo 3 mostrou que os dados do uso da biopsia líquida nesta área ainda são inconclusivos. Contudo, os níveis mais altos de fragmentos longos de cfDNA e o índice de integridade, as mutações específicas do tumor e alguns genes metilados foram preditores de não respondedores. Adicionalmente, ctDNA indetetável pré-tratamento e diminuição de mutações comuns no cancro do reto ao longo do tratamento (monitorização dinâmica) foram preditores de resposta patológica completa. Para além disso, a deteção contínua do ctDNA em diferentes pontos no tempo do tratamento (doença residual mínima) esteve associada a pior prognóstico. No artigo 4, embora os resultados devam ser lidos com cuidado devido à pequena amostra, sugerimos que o ctDNA pré-tratamento combinado com a invasão vascular extramural na ressonância magnética pode potencialmente ajudar a prever resposta, e que o número de mutações do ctDNA pré-tratamento pode ajudar a discriminar grupos com diferente sobrevivência livre de doença.

Conclusões

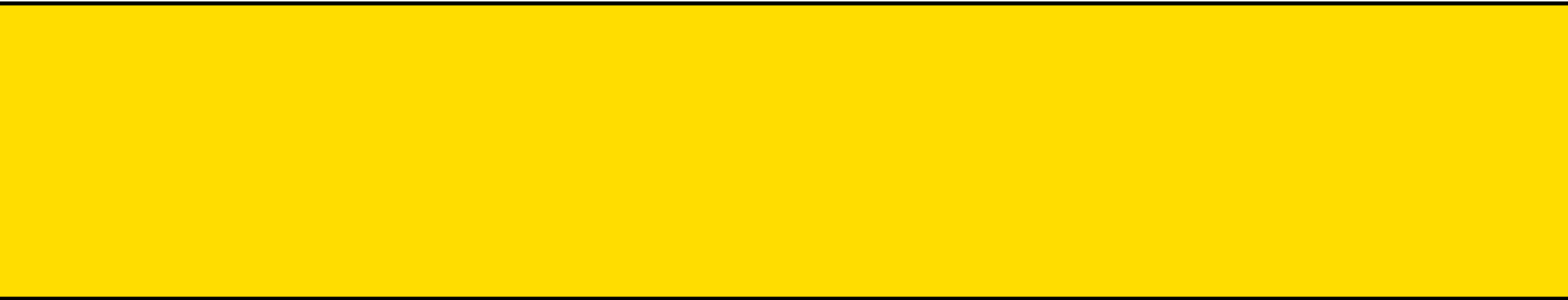
Esta tese contribuiu para melhorar a compreensão do valor dos biomarcadores plasmáticos na previsão de resposta ao tratamento neoadjuvante e prognóstico dos doentes com cancro do reto localmente avançado.

Os benefícios de identificar os doentes não respondedores poderá melhorar a sua qualidade de vida ao evitar exposição desnecessária a agentes citotóxicos. Também poderá sugerir que uma abordagem mais agressiva poderá ser mais apropriada nos doentes não respondedores. Para além disso, o aparecimento de estratégias de preservação de órgão poderá beneficiar de marcadores precisos e reprodutíveis

A construção de *scores* e nomogramas com marcadores hematológicos e bioquímicos em uso na prática clínica, poderia ajudar a sub-estadiar doentes de alto e baixo risco dentro de cada estágio TNM. Os métodos ideais e os tempos de colheita da biopsia líquida necessitam validação, mas por agora a análise seriada de mutações específicas do ctDNA nos tempos mais oportunos durante o tratamento, adicionalmente aos métodos de imagem atualmente usados, parece ser a forma mais eficaz para alterar decisões clínicas no futuro.

Contudo, a previsão de resultados no cancro do reto localmente avançado pode não ser possível apenas a partir de variantes isoladas específicas do tumor. O aparecimento de novos sistemas de sub-estadiamento alertam para a necessidade do estudo de perfis de expressão variável na previsão de resposta e prognóstico, e de que variantes específicas isoladas poderão não ser suficientemente importantes. A inclusão dos marcadores e mutações estudados nesta tese em sistemas de estadiamento poderá ajudar a estratificar o risco dos doentes e em última análise otimizar o tratamento, sobrevivência e qualidade de vida dos doentes com cancro do reto localmente avançado.

BACKGROUND



Background

1. Epidemiology

Colorectal cancer (CRC) is a large worldwide health problem, with 1.88 million new cases and 0.92 million new deaths reported in 2020 ¹¹, and with a burden expected to increase 60% by the year 2030 ^{12 13}. The majority of cases appear in developed countries, such as Western Europe, North America, New Zealand and Australia, while low incidence rates are reported in Asia, Africa and South America ¹⁴. In Portugal, 60467 cases have been diagnosed in 2020 and this disease represents the first cause of cancer in incidence, followed by breast and prostate cancer. A mortality of about 12% has been reported, making CRC the second cause of death by cancer ¹⁵. Approximately 25-30% of all CRC are located in the rectum ¹⁶.

There is a plethora of risk factors for CRC, such as hereditary factors, in which 20% are related with familiar genetic factors, with known causative germline alterations being present in around 5% ¹⁷; modifiable risk factors, where smoking, red meat, excessive alcohol consumption and obesity increase risk, and fish, whole grains, dairy products, fruit, vegetables and physical activity decrease risk. Other important risk factors include sociodemographic factors, like male sex and older age, and medical factors, such as diabetes and inflammatory bowel disease which increase risk, and large bowel endoscopy, aspirin, statins and hormone replacement therapy decreasing risk ^{18 19}.

2. Anatomy and Pathophysiology

Rectal cancers are clinically defined as tumors which arise within 15cm of the anal margin, whereas colon cancers are more proximal intestinal tumors. According to location, rectal cancers can be categorized as high (10-15cm from anal margin), middle (5-10cm) or low (<5cm) ²⁰.

The middle and low rectum are located below the peritoneal reflection and the distal limit is the dentate line, where the squamous mucosa of the anus alters to the columnar mucosa of the rectum. Contrasting with the colon, the rectum lacks taeniae, epiploic appendices, haustra and a well-defined mesentery. The mesorectum is the perirectal areolar tissue that supplies the rectum (Figure 1) and

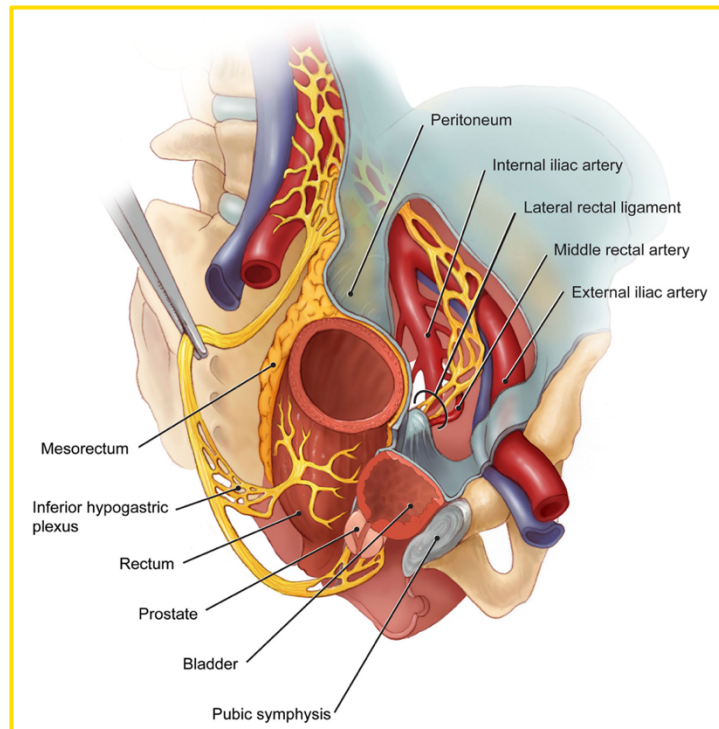


Figure 1. Hemisected pelvis showing the surrounding structures of the rectum (reprinted with permission from ⁹)

contains the terminal branches of the inferior mesenteric artery, the draining perirectal lymphatics and lymph nodes of the rectum ²¹. The main function of the rectum is to store the compacted fecal matter, product of the gastrointestinal tract, before defecation. The large intestine houses the largest number of intestinal bacteria (approximately 200 grams), most being commensals, meaning they do not induce disease ²². It is also believed that these microorganisms and their metabolic output play a role in CRC development, perhaps by inducing inflammation, DNA damage and apoptosis ²³.

3. Carcinogenesis and Progression

CRC carcinogenic evolution initiates as emergence of malignant cells in the epithelial layers of the bowel (stage 0), followed by invasion of these cells into the muscular layer (stage I). In stage II, the tumor penetrates the bowel wall into neighboring organs, and then spreads into the lymph nodes in stage III. When cancer cells spread throughout the body, it results in stage IV. This secondary spread usually spreads to the liver and lungs, but may also occur in bones, adrenals, lymph nodes, brain, skin and the oral anatomic sites ²⁴.

The progression through stages is originated by the progressive accumulation of genetic and epigenetic mutations that contribute to the histological transformation of normal colonic mucosa into adenocarcinoma. Two sequences have been described. The first is the adenoma-carcinoma sequence described by Fearon and Vogelstein, in which normal intestinal glandular epithelium transforms into tubular adenoma and then adenocarcinoma (Figure 2). The second describes a sessile serrated adenoma as a pre-neoplastic lesion ²⁵. The serrated neoplasia sequence is associated to different genetic and epigenetic changes from the traditional adenoma-carcinoma sequence ²⁶.

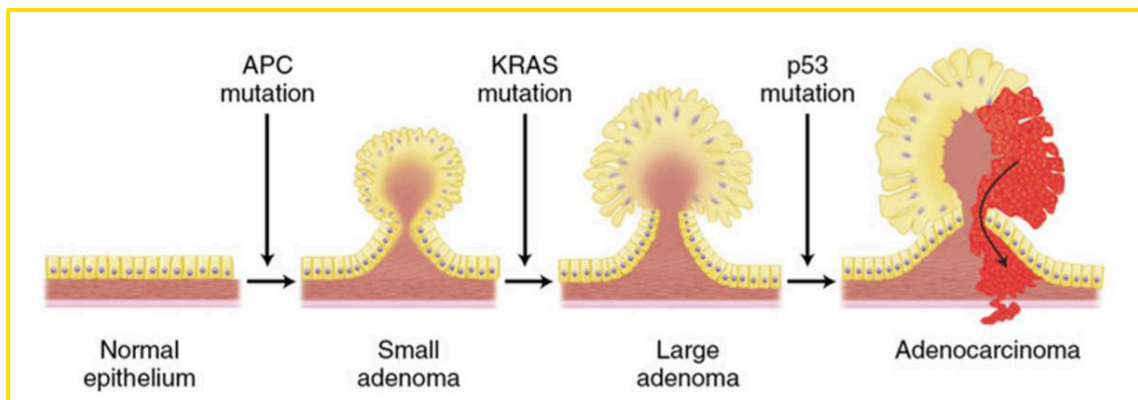


Figure 2. Main molecular and histological changes in the adenoma-carcinoma sequence (reprinted with permission from ⁷)

Three different pathways of genomic and epigenomic instability have been identified: chromosomal instability, microsatellite instability and epigenetic instability ²⁷.

BACKGROUND

In the carcinomas with chromosomal instability, the most common form of genomic instability, the alteration of the *APC* (adenomatous polyposis coli) / β catenin / *Wnt* pathway is a frequent early event in the adenoma-carcinoma sequence and is present in 70% of tubular adenomas. Functional deactivation of *APC* (an oncosuppressor gene) leads to uncontrolled activation of the *Wnt* pathway, resulting in increased transcription of β catenin target genes, which promote cell proliferation.

The next step in the “adenoma-carcinoma sequence” and still an early event of tumorigenesis is *KRAS* (Kirsten rat sarcoma viral oncogene homolog) mutation, which is mutated in about 40% of colorectal cancers and most frequently in codons 12 and 13. Ultimately, *KRAS* gene controls cell survival and proliferation processes and, as a protooncogene, is often overactive in CRC. This mutation leads to the constitutive activation of *RAF* (rapidly accelerated fibrosarcoma), *MAPK* (mitogen-activated protein kinases), independently from the binding of *EGF* (epidermal growth factor) to its receptor. This explains both the loss of efficacy of anti-EGFR (EGF receptor) monoclonal antibodies and the predictive role of response to therapy by the knowledge of *KRAS* gene mutation. In 32% the mutation affects the *PIK3CA* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), and more rarely the tumor suppressor *PTEN* (phosphatase and tensin homolog), which negatively regulates *PI3K* (phosphoinositide 3-kinases) ²⁸.

The development of adenoma from intermediate to the late stage is instigated by the loss of chromosome 18q (DCC – deleted in colorectal cancer) ²⁹ and it accounts for around 70% of advanced or late adenoma ³⁰. This loss also affects *SMD2* (S-adenosylmethionine decarboxylase proenzyme) and *SMAD2* (mothers against decapentaplegic homolog4), genes that encode proteins that act on TGF- β (transforming growth factor beta) receptors complex and play a crucial role in cell growth, differentiation and apoptosis ³¹.

The shift from advanced adenoma to adenocarcinoma frequently happens by loss of chromosome 17q in 50% of CRC cases. *TP53* (tumor protein p53) gene is an oncosuppressor gene that plays a critical role in the maintenance of genomic stability through control of the cell cycle G1 / S checkpoint. Mutations of this gene are present in up to 75% of advanced carcinomas and cause loss of function of its protein product – p53 ³². This gene is a target of 17qLOH (loss of heterozygosity)

and its mutation contributes to uncontrollable growth in the late phases of adenoma-carcinoma sequence^{33 34}.

There is an alternative genetic mechanism of tumorigenesis. MSI (microsatellite instability) is present in 15% of sporadic cases of CRC and it is caused by inactivation of DNA MMR (mismatch repair) genes such as *MLH-1* (mutL homolog 1) and *MSH-2* (mutS homolog 2). The hypermethylation of these genes leads to a hypermutable phenotype, due to the lack of ability to repair the short repeat (1-6 base-pair) of DNA sequence³⁵ and is characterized by genomic instability and progressive accumulation of mutations³⁶. DNA MMR-deficient tumors can originate in adenomas with active point mutations in *BRAF* (V-Raf murine sarcoma viral oncogene homolog B) oncogene, which drives the CIMP (CpG island methylator phenotype) and hypermethylation of promoter-specific sites of the genome. These tumors are characterized by normal chromosomal karyotype and associated with exclusive MSI³⁷.

4. Diagnosis and Staging

The diagnosis of CRC is based on clinical history and physical exam, digital rectal examination (DRE), fecal occult blood test, barium enema, sigmoidoscopy, colonoscopy and biopsy. Presenting symptoms include tenesmus, change of bowel habit, abdominal pain, rectal bleeding, weight loss and symptoms of anemia, such as fatigue, lethargy and shortness of breath ³⁸. After diagnosis through colonoscopy and biopsy, toraco-abdomino-pelvic computed tomography (CT) imaging is used to evaluate the extent of the disease, including distant metastatic spread. Magnetic resonance imaging (MRI) or endorectal ultrasound (ERUS) provide locoregional assessment of T stage, circumferential resection margin (CRM) and anal sphincter complex involvement, lymph-node involvement and vascular invasion ³⁹. The majority of rectal carcinomas (approximately 90%) are classified as adenocarcinomas, while the remaining represent mucinous carcinomas (9%) or some very rare epithelial tumor types (less than 1%), such as signet ring cell carcinomas, adenosquamous or undifferentiated carcinomas.

The TNM (tumor node metastasis) staging system has been standard to stage and guide treatment and prognosis of CRC. This system is based on primary tumor depth of invasion (T), lymph node involvement (N) and the presence of metastasis (M) and can be determined clinically (cTNM), through clinical examination and imaging or pathologically (pTNM), by evaluation of surgical specimens ²⁰. Table 1 displays the current staging (8th edition) of CRC, published by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) in 2016 ⁶. A change to the 7th edition is the addition of stage M1c, which denotes that peritoneal involvement carry significantly worse prognosis ^{40 41}. Another alteration related to the presence of micrometastases within regional lymph nodes (clusters of 10 to 20 tumor cells or clumps of tumor that measure ≥ 0.2 mm in diameter) as positive, as they preclude a higher likelihood of disease recurrence in stage I and II CRC, than isolated tumor cells ⁴².

In this work, we intend to focus primarily on patients with locally advanced rectal cancer (LARC), which is clinically defined as stage II/III.

Table 1. AJCC stage groups according to AJCC staging manual, 8th edition ⁶

T Stage (Primary tumour)	N Stage (Regional Lymph nodes)	M Stage (Distant Metastasis)	T Stage	N Stage	M Stage	Stage Group
Tx - Primary tumour cannot be assessed	Nx - Regional lymph nodes cannot be assessed	M0 - No distant metastasis	Tis	N0	M0	0
T0 - No evidence of primary tumour	N0 - Regional lymph node metastasis	M1 - Distant metastasis present	T1-2	N0	M0	I
Tis - Carcinoma in situ: intraepithelial or invasion of lamina propria	N1 - 1-3 regional nodes affected	M1a - Confined to one organ or site (e.g. distant lymph nodes, liver, lung)	T3	N0	M0	IIA
T1 - Invades submucosa	N1a - One regional node	M1b - More than one organ/site	T4a	N0	M0	IIB
T2 - Invades lamina propria	N1b - 2-3 regional nodes	M1c - Peritoneal metastasis	T4b	N0	M0	IIC
T3 - Invades muscularis propria into pericolic tissues	N1c - Tumour deposit(s) in subserosa, mesentery, or nonperitonealised pericolic or perirectal tissues without regional nodal metastasis		T1-2	N1/N1c	M0	IIIA
T4a - Penetrates to surface of the visceral peritoneum	N2 - 4 or more nodes		T1	N2a	M0	IIIA
T4b - Directly invades or is adherent to other organs or structures	N2a - 4-6 regional nodes		T3-T4a	N1/N1c	M0	IIIB
	N2b - 7 or more regional nodes		T2-3	N2a	M0	IIIB
			T1-2	N2b	M0	IIIB
			T4a	N2a	M0	IIC
			T3-4a	N2b	M0	IIC
			T4b	N1-2	M0	IIC
			Any T	Any N	M1a	IVA
			Any T	Any N	M1b	IVB
			Any T	Any N	M1c	IVC

The MERCURY (MRI and Rectal Cancer European Equivalence) study group ⁴³ further divided the cT3 stage in 4 categories and different treatments for each of these were suggested. In this classification, cT3a tumors with less than 5mm of intrusion into the perirectal fat and predicted negative resection margins generally behave more as T2 lesions and thus could be spared the negative consequences of radiation therapy ⁴⁴. For cT3 lesions with close but predicted negative (>2mm) margin, either short-course or long-course radiotherapy could be considered. For cT3 lesions with predicted positive margin or cT4, long-course chemoradiotherapy is required for tumor downstaging.

Still regarding subclassification of CRC, MSI-high/MMR-deficient (dMMR) patients with CRC stage II were recently reported to have a better prognosis than MSI-low patients and to not benefit from 5-fluorouracil-based adjuvant therapy ⁴⁵. However, although about 24% of all proximal colon tumors were reported to have MSI, only 2% of rectal tumors were MSI positive ⁴⁶. This fact might help explain why rectal cancer often have poorer prognosis than colon cancer patients, with higher risk of distant metastasis ⁴⁷. Furthermore, the fact that MSI-high and dMMR tumors are much rarer in patients with rectal than colon cancer, may limit its usefulness in these patients ⁴⁸.

The TNM staging system is currently the most widely implemented method of prognosis in CRC. However, some criticism has been growing towards the TNM paradigm by precision medicine research, in which specific biomedical information

of a patient has been showing to make more accurate diagnosis, personalization of treatment and better prediction of prognosis.

Historically, the TNM staging system implied a sequential progression from local invasion through lymph node involvement to distant metastasis. A different so-called “parallel invasion model”^{49 50} explains that metastases can arise from tumor cells that disseminate early in the development of the primary tumor, through hematogenic route⁵¹. By bypassing the regional lymph nodes, they can progress in parallel to the primary tumor⁴⁹. This model is supported by genetic studies⁴⁹ where samples from the primary tumor, lymph nodes and distant metastases of individual patients are genetically distinct⁵².

A further drawback of the TNM staging system in CRC is the heterogeneity of outcomes inside the same TNM stage⁵³, for instance 5-year survival in patients with stage II colon cancer can range from 87.5-58.4%, depending on T stage⁵⁴. Another argument is the so-called “survival paradox” between stage II and III CRC⁵⁵ found in a South Korean study of 5547 patients, in which patients with IIA/B disease demonstrated a worse overall survival (OS) and disease-free survival (DFS) compared with IIIA disease, despite an even distribution of adjuvant chemotherapy and lymph node sampling between the groups.

4.1. Future Methods of Clinical Staging

The improvement of current stratification could allow the incorporation of molecular biomarkers already available for risk stratification and evaluation of targeted therapies. The investigation on gene-expression of the Colorectal Cancer Subtyping Consortium (CRCSC) have suggested a novel stratification for CRC consisting of four Consensus Molecular Subgroups (CMS)⁵⁶, with different characteristics and biological behavior:

- CMS1 (MSI-immune, in 15%; characterized by microsatellite instability hypermethylation, immune cell infiltrates and upregulated immune checkpoints).
- CMS2 (canonical, in 35%; characterized by the overexpression of *WNT* and *MYC* signal-dependent pathways).

- CMS3 (metabolic, in 15%; characterized by metabolic dysregulation).
- CMS4 (mesenchymal type, in 24%; with overexpression of factors derived from mesenchyme-regulating angiogenesis and stromal invasion and with the worse relapse-free and overall survival).
- The remaining (about 10%; can be defined by mixed characteristics).

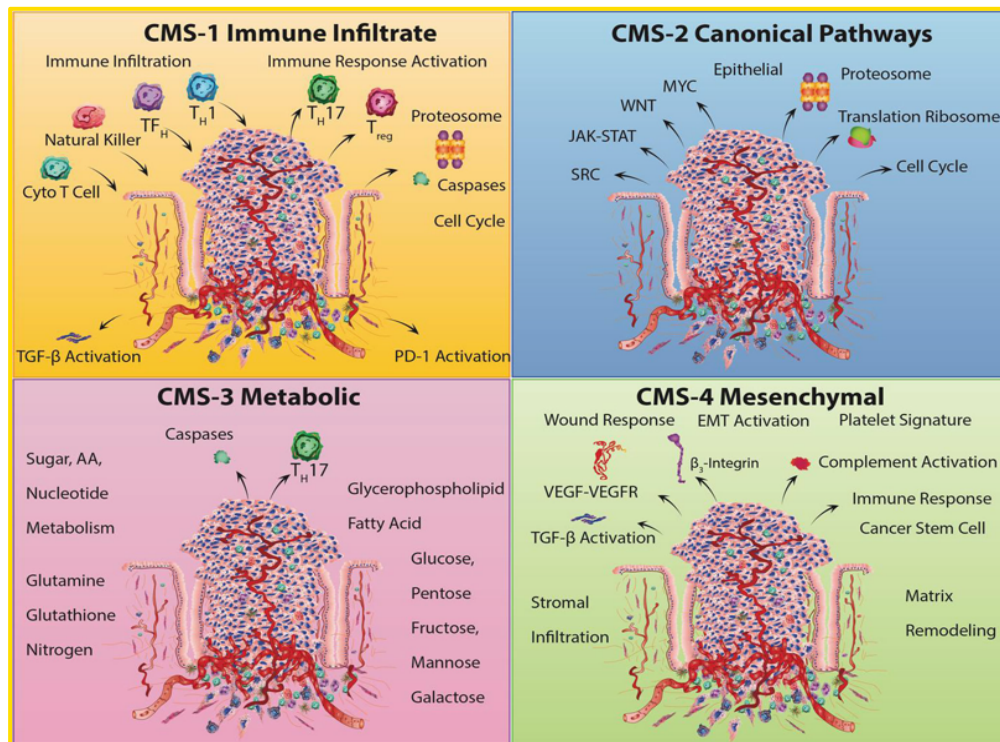


Figure 3. The consensus molecular types (reprinted with permission from ²⁾)

Bertotti et al. ⁵⁷ also investigated cancer cell intrinsic transcriptional traits and found 5 CRC intrinsic subtypes as well, defined as CRIS-(A-E), using the same biomarkers.

5. Current Management of Patients with LARC

All patients should be discussed in a multidisciplinary meeting comprising surgeons, pathologists, radiologists and medical oncologists. For LARC patients, standard treatment is curative surgery, after pre-operative radiotherapy, with or without concurrent chemotherapy ²⁰, in order to downstage the tumor. This also allows sphincter-saving procedures, which improve patients' quality of life ⁵⁸. Upper rectal cancers (>12 from the anal verge) usually do not benefit from neo-adjuvant treatment, due to their better prognosis and lower incidence of local relapse ⁵⁹, and will not be discussed in this thesis.

5.1. Surgery

The aims of surgery are to achieve local disease control, avoid local recurrence and improve long-term survival ²⁰. Radical surgery of the rectum with total mesorectal excision (TME), described by Heald in 1979 ⁶⁰, has become the standard surgical technique for patients with LARC. A curative complete resection with microscopically margin-negative resection (R0) consists of anterior resection of the tumor-bearing bowel segment with adequate proximal (5cm), distal (1cm) and circumferential (> of 1mm) resection margins ^{61 62}, central ligation and division of the supplying blood vessels ⁶³. Patients with fecal continence and a rectal tumor in the low or mid rectum with no sphincter involvement most often undergo a low anterior resection. When low-third rectal cancer involves the sphincters or has pelvic fixation, an abdominoperineal excision is indicated, with four options available (intersphincteric, conventional, extralevator and ischioanal) ⁶⁴. These result in a permanent stoma, with concurrent complete removal of the anal sphincter complex ⁶⁵. However, in recent years, sphincter-saving surgery has gained recognition ⁶⁶. A TME corresponds to the removal of the mesorectal fat and lymph-nodes surrounding the primary tumor, with sharp dissection of distinct avascular embryological planes to avoid breaching the visceral layer, which has shown to reduce risk of local recurrence ^{67 68}. Heald achieved local recurrence rates as low as 3.7% ⁶⁹.

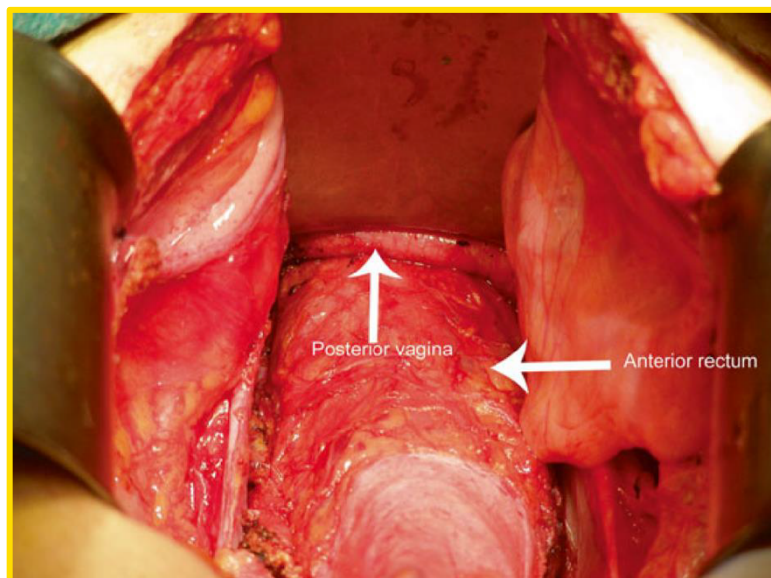


Figure 4. Intra-operative photograph of an anterior rectum resection, demonstrating a TME dissection plane in a female patient (reprinted with permission from ⁸)

Laparoscopic surgery has been proven safe in these patients, with no difference in long-term results and with better short-term outcomes ⁷⁰, such as lower intraoperative blood loss, earlier resumption of food intake, lower 30-day postoperative mortality rate, reduced hospital stay and cost-saving when employed in selected patients or performed by experienced surgeons in high volume centers ⁷¹⁻⁷³.

5.2. Neoadjuvant Treatment

5.2.1. Radiotherapy

In the 1990s, the Swedish and Dutch rectal cancer clinical trials have demonstrated that pre-operative radiotherapy followed by surgery had a benefit in local control and survival compared to LARC patients who only received surgery ⁷⁴ and also in comparison to postoperative combined modality therapy ⁵⁸. In recent years, a systematic review has confirmed these results, with a reported better overall survival and local control, although no difference was observed in disease-specific mortality. Interestingly, patients who received TME did not have the same benefit in overall survival after short course radiotherapy, which might have to do

BACKGROUND

with recent improvements in surgical techniques ⁷⁵. A possible explanation for the advantage of neoadjuvant in comparison with adjuvant radiotherapy is that ionizing radiation is more effective in virgin tissue due to increased oxygen tension in the tissue, instead of hypoxic post-surgical tissues. Other advantages include less radiation-induced small bowel injury in the pelvis, which are not fixed by previous surgery and the possibility to excise the irradiated rectal segment and perform an anastomosis to healthy non-irradiated colon ⁵⁸.

Two modalities of administration of radiotherapy can be applied to these patients: a long-course which exposes the patients to low doses (1.8-2Gy per dose/fraction) over a 5-week period (total 45-50Gy) and a short-course radiotherapy which involves much higher doses (5Gy per dose/fraction) but over a shorter period of 1 week (total 25Gy) ²⁰. Some studies reported no differences in local control or survival between both methods ⁷⁶. Nowadays, there are no guidelines for deciding which radiotherapy course to use but patient frailty and co-morbidities have to be taken into account in this decision. Short-course regimen has been the preferred treatment in Northern Europe, Scandinavia, North America and some European countries, however long-course 5-FU-based (5-fluorouracil) chemoradiotherapy has become the elected treatment with time in Western countries, with a 6-8 weeks delay before surgery ^{77 78}. Lower early toxicity, better compliance and lower cost make short-course radiotherapy a suitable option for elderly high-risk patients ⁷⁷. As for long-course chemoradiotherapy, its higher rate of tumor downstaging and complete pathological response (pCR) make it more valuable for high-risk patients, such as low tumors and when mesorectal fascia is threatened ⁷⁹.

By improving the chances of an R0 resection and decreasing rates of local recurrence, radiotherapy can improve long-term quality of life. Nevertheless, radiotherapy is also associated with short-term toxicity in 50% of patients and significant long-term side effects, such as fibrosis, autonomic nerve injury (manifested by bowel, bladder and sexual dysfunction), infertility, uterine incompetence and diminished hematopoiesis due to irradiation of pelvic bone marrow ⁸⁰. Furthermore, in parallel, clinical studies have attempted to improve patient outcomes through the addition of chemotherapy.

5.2.2. Chemotherapy

Pre-operative radiotherapy may be administered with concurrent chemotherapy. The standard agent used in LARC patients is capecitabine (an orally administered fluoropyrimidine which is metabolized into 5-FU within tumor cells). Capecitabine has a radiosensitizing nature in tumor cells and improves tumor response to radiotherapy, although no effect is seen in untargeted locoregional lymph-nodes or distant metastasis ⁸¹. Fluorouracils inhibit thymidine synthase, block the production of thymidylate and the downstream cellular DNA synthesis ⁸². These drugs present a narrow therapeutic index and around 15-30% of patients suffer from severe toxicity, presenting diarrhea, nausea, mucositis, stomatitis, myelosuppression, neurotoxicity and hand-foot syndrome. Moreover, a mortality of 0.5-1% has been reported ⁸³. Moreover, other cytotoxic agents, such as oxaliplatin, irinotecan and leucovorin, could theoretically enhance the cytotoxic effects of fluorouracils. However, some trials have found greater toxicity with no improvement in outcomes ⁸⁴.

Studies have shown that the addition of concurrent chemotherapy to pre-operative radiotherapy improved pathological response and local control in LARC patients, although no effect on disease-free survival or overall survival was achieved ^{85 86}.

Furthermore, other targeted therapies, some already used in the metastatic CRC setting, are under research, but have not shown clear benefits to this date. These include anti-EGFR monoclonal antibodies (cetuximab ⁸⁷ and panitumumab ⁸⁸) in *KRAS* wild-type cancers, immune checkpoint (PD-1) inhibitors (pembrolizumab and durvalumab), anti-angiogenic monoclonal antibodies (bevacizumab ⁸⁹ or cediranob) and poly-ADP ribose polymerase (PARP) inhibitors (veliparib).

5.3. Tumor Pathological Response and Organ Preservation Strategies

After neoadjuvant treatment, approximately 10-20% of patients have pCR, where the tumor has been eliminated in the specimen ⁹⁰. This is achieved when surgery is delayed more than 5-12 weeks after neoadjuvant chemoradiotherapy

BACKGROUND

(nCRT) ⁹¹⁻⁹³, although maximal tumor response after nCRT can take up to 26 weeks ⁹⁴. Also, 60% can attain downstaging of the tumor ⁹². This may facilitate surgery, allowing sphincter preservation ⁹⁵ and potential clearance of microscopic tumor spread, safely permitting a closer distal margin at resection ^{96 97}. The remaining patients can experience variable outcomes, from good response with plenty of regression, to minimal or no response. Tumor regression grade (TRG) systems are applied to classify response of the tumor to nCRT. Several TRG systems are available, including Dworak's ⁹⁸, Mandard's ⁹⁹, Ryan's ¹⁰⁰, AJCC ¹⁰¹, and Memorial Sloan Kettering Cancer Center (MSKCC) ¹⁰², as seen in Table 2.

Table 2. Tumor regression grade systems (reproduced from ¹)

	Dworak	Mandard	Ryan	AJCC
Complete regression	No tumor cells (TRG 4)	No residual cancer cells (TRG 1)	No viable cancer cells, or single cells, or small groups of cancer cells (TRG 1)	No viable cancer cells (TRG 0)
Near complete regression	Very few tumor cells (TRG 3)	Rare residual cancer cells (TRG 2)	-	Single or small groups of tumor cells (TRG 1: moderate response)
Moderate regression	Dominantly fibrotic changes with few tumor cells or groups (TRG 2)	Predominant fibrosis with increased number of residual cancer cells (TRG 3)	Residual cancer outgrown by fibrosis (TRG 2)	Residual cancer outgrown by fibrosis (TRG 2: minimal response)
Minimal regression	Dominant tumor mass with obvious fibrosis (TRG 1)	Residual cancer outgrowing fibrosis (TRG 4)	Significant fibrosis outgrown by cancer, or no fibrosis with extensive residual cancer (TRG 3)	Minimal or no tumor cells killed (TRG 3: poor response)
No regression	No regression (TRG 0)	No regressive change (TRG 5)	-	-

Patients who achieve pCR show a decreased likelihood of experiencing local recurrence ¹⁰³ and an improved DFS ¹⁰⁴. Therefore, an alternative option to surgery after neoadjuvant treatment has been emerging, in order to avoid the long-term sequelae of such a radical surgery, including urinary and sexual dysfunctions, fecal incontinence, temporary or permanent stomas; and also avoid its immediate post-operative risks, bleeding, infection and anastomotic leak ¹⁰⁵. The “watch and wait” approach was popularized by Habr-Gama et al. ¹⁰⁶ in 2004, for patients who achieve complete clinical response (cCCR). These patients do not receive any surgery and are maintained under close surveillance. cCR is defined by the European Society of Clinical Oncology (ESMO) as the absence of the any palpable tumor during DRE, no visible tumor at proctoscopy and absence of detectable tumor at primary site or in lymph nodes through MRI or ERUS. Surveillance is performed using these methods, because no current biomarkers have been proven to predict the response to nCRT.

Interestingly, cCR and pCR still appear to have poor concordance in patients with rectal cancer ¹⁰⁷. Nevertheless, Renehan et al. (2016) ¹⁰⁸ have reported a similar DFS at 3 years for both approaches (“watch and wait” vs. surgery). But, alarmingly, a wide variability in local recurrence rate has been noticed for the “watch and wait” approach (5-60%), which may be due to the heterogeneity of studies on follow-up time and surveillance intensity. In a systematic review of nine studies, persistent cCR was achieved in 69.2% (256/370 patients) and in those with tumor regrowth, salvage surgery was possible in 83.8% ¹⁰⁹. Despite the promising results, this approach has not been widely implemented and research groups continue to analyze its oncological safety. This strategy may be considered in centers with experienced multidisciplinary teams (MDT) after a careful discussion with the patients about their risk tolerance ⁹².

5.3.1. Current Predictors of Response

In order to stratify patients and provide personalized treatments, studies have been focusing on factors that can predict response to nCRT. Currently, clinicians rely on pre-operative staging scans and the treatment is decided based on the clinical features of the tumor (cTNM) ³⁹. Nevertheless, TNM stage has not been proved to be a significant predictor of response to nCRT. Therefore, several factors have to be taken into account:

- Clinical and endoscopic evaluation: DRE, endoscopy and biopsy play an essential role in the assessment of cCR ¹⁰⁹ after nCRT. The limitation of DRE is the discordance between assessment and pathological response, and in a study only 21% of pCR were correctly identified ¹¹⁰. As for endoscopy and biopsy, only 59% of pCR were diagnosed correctly ¹¹¹, which could be due to the fact that residual rectal cancer is mostly located in deeper layers of the bower wall and biopsy results were unreliable ¹¹². Furthermore, neither DRE nor endoscopy are able to assess nodal involvement after nCRT.
- Imaging studies: these include ERUS, MRI and positron emission tomography (PET). Owing to inflammation, necrosis and desmoplastic changes after nCRT, the overall ypT stage prediction has low accuracy ^{113 114}, between 43-

73%. However, ERUS may have a role in confirming lymph node negativity after pre-operative treatment, with negative predictive values that could reach 81-88% ^{111 115}.

The utility of MRI in re-staging to assess pCR has been investigated by 2 groups (Regina Beets-Tan and Gina Brown), and due to the same limitations of ERUS ^{116 117}, accuracy for assessing T and N stage were only 45-67% and 65-75%, respectively ¹¹⁸. The MERCURY group developed the MRI TRG (mrTRG) system, based on the pathological TRG, to stratify good and poor responders ¹¹⁷ and they also discovered that extra-mural venous invasion (EMVI) was significantly predictive of poor responders ¹¹⁹. Nevertheless, the accuracy of this tool to predict pCR is uncertain, because patients were stratified as good versus poor responders. In a large meta-analysis (1566 patients from 33 studies), applying diffusion weighted imaging could raise sensitivity to 84% but it lowered specificity of MRI from 94% to 85% ¹²⁰, with heterogeneous results. Thus, MRI is not completely reliable to dictate non-surgical rectal conserving approach.

PET allowed the differentiation of responding and non-responding tumors with an overall accuracy of 80% ¹²¹. When researchers decreased mean standardized uptake value (SUV), higher accuracies were observed ¹²². However, there is still a limited number of studies, no cut-off mean value for SUV has been established and false positive tests have been reported ¹²³.

- Combination of clinical evaluation and imaging studies: this combination has shown increased detection of pCR ^{116 124} to rates of 96-98%. Nevertheless, randomized trials are lacking to confirm these results, although the TRIGGER trial (mrTRG as biomarker for stratified management of rectal cancer patients) ¹²⁵ is currently studying the utility of mrTRG in patient response stratification.
- Laboratory analysis: two different markers, routinely performed in clinical work-up, have been consistently associated with pCR: CEA (carcinoembryonic antigen) ^{126 127} and NLR (neutrophil-to-lymphocyte ratio) ¹²⁸. The major limitation to apply this in clinical setting is that most studies have discrepancies in the mean cut-off points used, which should be assessed in larger multicenter studies.

- Genomics: some gene expression panels are available for defining prognosis and decision of adjuvant treatment in stage II and III colon cancer, such as Oncotype Dx[®] colon cancer test ¹²⁹, ColoPrint[®] ¹³⁰ and ColDx ¹³¹. To date, there is still no relevant data on these panels for LARC. However, some genetic and molecular markers have been implicated in response to nCRT, such as EGFR, thymidylate synthase genes, bcl-2/bax (B-cell lymphoma protein 2-associated X) and COX-2 (cyclooxygenase 2) ¹³², although none has been robust enough for clinical application.

5.4. Adjuvant Treatment

The standard treatment mentioned above has decreased 70% of loco-regional recurrences, however it has failed to improve overall survival and metastatic recurrence, which is the most frequent cause of death in rectal cancer patients ¹³³. Over the years, studies have reached inconclusive results on the benefit of adjuvant treatment in these patients and international recommendations (NCCN – National Comprehensive Cancer Network, and ESMO – European Society for Medical Oncology) ^{92 134} are not consensual, mostly due to heterogeneity of inclusion criteria (the use of either cTNM or ypTNM classifications), inclusion of high rectal tumors, difficulties in trial recruitment, lack of statistical power and variations in preoperative and adjuvant regimens ⁹². Currently, adjuvant chemotherapy with a 5-FU regimen is usually recommended in patients who are node negative in the resected specimen after neoadjuvant therapy. Patients who reached yp stage I tumor or histological complete response do not benefit from adjuvant treatment. Contrastingly, FOLFOX (5-FU, leucovorin and oxaliplatin) chemotherapy regimen should be offered to yp stage III patients and considered for yp stage II tumors in fit patients with high-risk factors, such as perforation, obstruction, lymphovascular or perineural invasion ¹³⁵. The treatment can be initiated within five weeks after surgery or when the patient is fit to receive therapy ¹³⁶. Despite these recommendations, a meta-analysis from 2015 found that adjuvant chemotherapy did not improve OS, DFS or distant recurrences, calling these recommendations into question ¹³⁷. Also, up to 27% of eligible patients never start adjuvant chemotherapy

and less than 50% ¹³⁸ receive the full prescribed treatment without interruptions or delay ¹³⁹ due to postoperative complications, slow recovery, interference with closure of their temporary ileostomy ¹⁴⁰ or treatment refusal ¹⁴¹. Furthermore, it has been proven that each 4-week delay in treatment correlated with a 14% decrease in OS ¹⁴².

This area will continue to evolve as evidence for preoperative chemotherapy strategy becomes available, and thus the benefit of chemotherapy in the postoperative setting may not persist in patients already exposed to doublet or triplet chemotherapy in the neoadjuvant setting.

5.5. Novel Therapeutic Options for LARC

Another potential emerging approach is to deliver all chemotherapy upfront, the so-called total neoadjuvant therapy (TNT), which has the potential advantages of treating occult micrometastases several months earlier, increasing treatment compliance, enhancing the efficacy of chemotherapy and ultimately improving survival ¹⁴³. Other benefits include increased response of primary tumor, early identification of non-responders and earlier removal of the loop ileostomy. Induction chemotherapy administered before chemoradiation and proctectomy is now considered a valid alternative to the standard approach, as a study from MSKCC found a tumor response greater than 90%, including 30% of cCR ¹⁴⁴. Chemotherapy can also be delivered as consolidation (after chemoradiotherapy completion and before surgery), without any associated increase in adverse events or surgical complications ¹⁴⁵. Also, in the most recent edition of the NCCN guidelines, TNT is already contemplated as an option in the treatment of LARC patients, and gradually these approaches are starting to be implemented in the clinical setting by many hospitals.

The recent Swedish study RAPIDO (Rectal cAncer and Preoperative Induction therapy followed by Dedicated Operation), published in 2020, tested a group submitted to neoadjuvant short-course radiotherapy followed by chemotherapy (6 cycles of capecitabine plus oxaliplatin, CAPOX and 9 cycles of FOLFOX) and then surgery (2-4 weeks after), against long-course nCRT followed by

surgery (6-10 weeks after) and adjuvant chemotherapy. They concluded that the first approach had benefits in DFS, lowered the risk of distant metastasis ¹⁴⁶ and significantly improved pCR (28% vs. 14%). Also in 2020, the results of PRODIGE23 study ¹⁴⁷, in which a group submitted to TNT (6 cycles of FOLFIRINOX - 5-FU, leucovorin, oxaliplatin and irinotecan) followed by nCRT and surgery after 6-8 weeks against conventional nCRT, showed similar outcomes to RAPIDO, with a pCR of 27.5%.

Furthermore, the preliminary results from the phase II OPRA trial (Organ Preservation in Rectal Adenocarcinoma), NCT 02008656, suggest initiating treatment with nCRT and then systemic chemotherapy (FOLFOX or CAPOX) (consolidation TNT vs induction TNT) may improve organ preservation (58% vs. 43%) ¹⁴⁸. There was no statistically difference in 3-year DFS between both arms, although a longer-term follow-up is required to assess the risks and benefits of this approach. Moreover, when patients demonstrate adequate clinical response to induction systemic chemotherapy, omitting radiotherapy and its toxicity might be a possibility (PROSPECT trial phase II/III NCT01515787) ¹⁴⁹.

Interestingly, for patients with dMMR (9% of rectal cancers ¹⁵⁰), neoadjuvant immunotherapy with programmed cell death 1 (PD-1) blockade is being investigated before standard nCRT and surgery, in hopes it might demonstrate improved response rates. Promising results have been seen with neoadjuvant immunotherapy in colon cancers ¹⁵¹, and the same is hoped to be extended to the rectal cancer population.

Another strategy for increasing pCR is radiotherapy dose intensification, which can be achieved by local boost therapy through intensity-modulated radiation therapy (IMRT) or contact/intraoperative radiation therapy (IORT), which has the advantage of increasing the delivered dose to the tumor without increasing toxicity to uninvolved surrounded bowel ¹⁵², especially for patients with T4 cancers ⁹². In a randomized trial, an endo-cavitary boost of 85 Gy increased complete or near-complete sterilization (57% vs. 34%), in comparison to external beam radiotherapy to 39 Gy ¹⁵³. However, lymph nodes may remain unaddressed with this technique. There are still few prospective studies on these techniques, therefore the clinical

effect and optimal use has to be defined in future studies for subgroups who might benefit.

6. Survival and Follow-up

As mentioned before, TNM staging strongly impacts the prognosis of CRC patients, as the outcomes diverge for each stage. While stage I and II CRC patients have an excellent overall 5-year survival rate 90.3% and 82% respectively, the survival declines to 70.4% for individuals with regional metastases to the lymph nodes (stage III) and dramatically declines to 12.5% in patients with distant metastasis^{19 154}. Making an early diagnosis is still a challenge, with about 30% of patients already presenting distant metastases at the time of diagnosis¹⁵⁵. Fortunately, European mortality rates have decreased over the first decade of this century by 15% in men and 13% in women, which is likely associated with treatment improvements and earlier detection from intensive screening¹⁵⁶. Whatsoever, even with early diagnosis, about 30% of patients still develop metastases¹⁵⁷. Pulmonary metastases are more frequent in rectal cancer, due to its vascular drainage going straight to the vena cava, whereas in colon cancer liver metastases are predominant, because of its vascular drainage through portal circulation⁶⁰.

Within the TNM stage, the outcome also varies significantly, for which other prognosis factors have to be taken into account³⁶. Therefore, the most widely established additional clinical-pathological prognostic factors include:

- Emergency presentation, with perforation, bleeding or obstruction exhibits poorer five-year survival compared with non-emergent presentation¹⁵⁸.
- The clinical condition of the patient at diagnosis and throughout the treatment, measured by performance status indices might also affect the prognosis¹⁵⁹.
- Histological grading, evaluated through the glandular content. Poorly differentiated or undifferentiated tumors are associated with worse outcomes³⁶. A disadvantage to clinical application is its inter-observer variability¹⁶⁰.

- Mucinous (when more than 50% of a lesion is composed of extracellular mucin) and signet-ring types are associated with worse prognosis and poor response to chemotherapy ¹⁶¹.
- Lympho-vascular ¹⁶² and perineural invasion ¹⁶³ are well-established factors of worse prognosis.
- A lymph node yield of at least 12 is thought to represent an optimal mesenteric resection and accurate lymph node staging by AJCC ⁶. The lymph node ratio (the ratio of number of positive lymph nodes by the total lymph node yield) has been found to be an independent predictor of overall and disease-free survival superior to lymph node yield ¹⁶⁴.
- Isolated tumor deposits are nodules within perirectal tissue away from the leading edge of the tumor and with no evidence of residual lymph node and considered part of N stage, due to differentiation from discontinuous tumor spread, totally replaced lymph node and venous invasion. They are associated with poorer OS and DFS ¹⁶⁵.
- Tumor budding: a single cell or cluster of ≤ 4 cells detected at the advancing edge of the invasive carcinoma. The total number of buds should be reported in pathological exam. It has been shown to be an independent prognostic factor in CRC ¹⁶⁶.
- CRM and TRG following neoadjuvant treatment in rectal cancer. A distance of 1 mm or less between the tumor and CRM has been shown to predict higher local and distant recurrence ¹⁶⁷. Worse TRGs are also associated with worse prognosis ³⁶.
- Risk assessment models: there is a shift toward delivering personalized treatment and thus, multi-variable risk assessment models have been developed to predict prognosis in colorectal cancer. In 2011, Valentini et al. ¹⁶⁸ created a multivariate nomogram to predict 5-year local recurrence distant metastases and OS in LARC patients. The variables used for the risk assessment model of distant metastases were N stage, T stage, surgical procedure and administration of adjuvant chemotherapy.

After establishing the prognosis and treating adequately, these patients must be followed for detection of recurrence and symptoms. In the follow-up of patients

BACKGROUND

treated for LARC, the guidelines recommend regular outpatient consultations once every 3-6 months within 2 years after curative surgery and once every 6 months to 1 year in the third to fifth years. The follow-up includes regular blood tests, with CEA and CA 19-9 (carbohydrate antigen 19-9), chest and abdomino-pelvic CT scan. Also, they recommend total colonoscopy and then every five years ^{20 169}.

CEA, a high molecular weight glycoprotein, was discovered by Gold and Freedman in 1956. It is secreted by epithelial tumor cells in the digestive tract and is a widely used prognostic tumor marker in CRC patients ¹⁷⁰. Increasing CEA levels after surgery can predict recurrence ¹⁷¹ and also correlate with tumor burden. Thus, abnormal CEA levels are rarely found in patients with tumor sizes smaller than 3cm ¹⁷² and is rarely used in the diagnosis of CRC ¹⁷³. Contrary to clinical practice paradigm, a Cochrane review from 2015 included 53 studies and concluded that CEA alone was insufficiently sensitive to detect post-operative recurrences in CRC ¹⁷⁴.

CA 19-9, a high molecular weight glycoprotein, may show increased levels in metastatic CRC (mCRC). Although not a specific marker for CRC, used together with CEA, it could raise their diagnostic sensitivity ¹⁷⁵. They are both biomarkers for late-stage disease or can be used to monitor mCRC patient response ¹⁷⁶.

Nevertheless, the recently published COLOFOL¹⁷⁷ and GILDA¹⁷⁸ randomized trials have found that intensive surveillance with the mentioned methods does not prolong survival in patients with nonselected colorectal cancer. This seems to preclude that only high-risk subgroups could benefit from a more intensive surveillance and that novel biomarkers could aid in their definition.

7. Novel Biomarkers for Prediction of Response and Prognosis

The clinical assessment of treatment response is the greatest challenge and limiting factor for safe implementation of the “watch and wait” approach. The concordance between clinical and pathological evaluation has traditionally been poor in terms of sensitivity (around 25%) for detecting pCR, and in terms of specificity (around 60-90%) for excluding residual disease ^{110 179}.

Biomarkers are specific biological indicators or markers of disease ¹⁸⁰, which are widely used in diagnosis, therapy and prognosis of a number of human diseases. Proteins are the main executor of biological functions and have been the most studied biomarkers ¹⁸¹, but DNA and RNA markers are acquiring notoriety. According to biomarkers’ application in the clinical setting, they can be applied in three categories: diagnosis, treatment and prognosis ^{182 183}.

Until March 31th, 2022, there were 10942 papers in PubMed concerning rectal cancer biomarkers studies, and the papers have been significantly increasing every year.

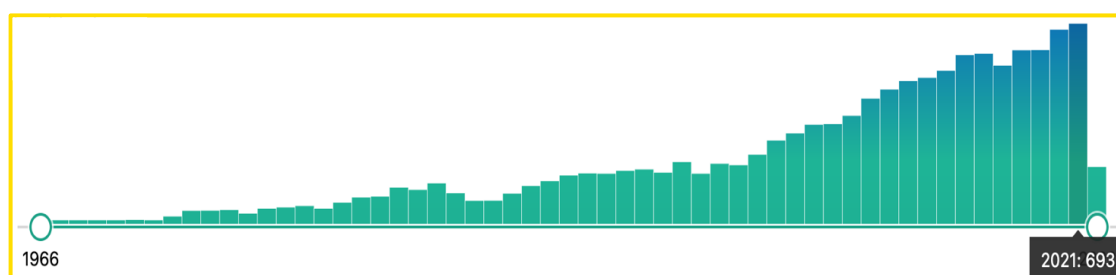


Figure 5. Distribution of paper count per year on Pubmed concerning biomarker research.
Search query: (biomarker OR marker OR predictor) AND (rectal cancer)

There are still a limited number of biomarkers which can facilitate decision-making for treatment of patients with LARS. Currently, only imaging tumor response and post-treatment circulating CEA levels are clinically used. The significance of pathological features and tumor regression grade was discussed above.

Subsequently, we review tissue- and blood-based biomarkers (retrieved through tissue and liquid biopsy, respectively) of growing interest in the literature ¹⁸⁴. Table 3 describes the characteristics, advantages and disadvantages of tissue and liquid biopsy of biomarkers.

Table 3. Comparison between tissue and liquid biopsy for analysis of biomarkers.

Tissue Biopsy	Liquid Biopsy
Allows histological diagnosis and staging	Does not allow tumor histotype
Often difficult and invasive	Non-invasive procedure
Not always representative of the entire diversity of malignant clone	Representative of different location of the malignant clones: tumor heterogeneity
Multiple sampling not always feasible	Easily repeatable and highly reproducible
Single snapshot over time and space	Real-time monitoring of disease
Still the gold standard for tumor characterization	Lack of standardization, still used mainly in translation research

7.1. Tissue-Based Biomarkers

Tumor Infiltrating Lymphocytes (TIL)

Tumors have the ability to suppress immune response by acquiring features to escape detection or through activation of negative regulatory pathways that are called “checkpoints”¹⁸⁵. In recent years, research has progressed towards clinical implementation of immunotherapies that act on these checkpoints, in patients with several cancer-types. Immune-related biomarkers have also begun to emerge as prognostic and response markers in patients treated with those drugs.

Previous reports have demonstrated that high densities of CD8+ (cluster of differentiation) cytotoxic T-lymphocytes were associated with improved response¹⁸⁶ and survival¹⁸⁷ after nCRT in rectal cancer patients. In LARC patients, high CD8+ TIL density was predictive of better tumor regression after nCRT¹⁸⁸ and greater sensitivity to nCRT¹⁸⁶. As for CD4+ TILs, some studies showed improved response¹⁸⁹, while another had contradictory findings¹⁹⁰. Huang et al. (2019) also demonstrated that location of TILs, specifically intraepithelial CD45+ and CD8+ were associated with improved response to nCRT while stromal TILs showed no association in LARC patients¹⁹⁰.

Studies have also reported that counts of CD8+ or FoxP3+ (forkhead box P3) regulatory T-cells were not significant prognosticators of outcomes, but changes in the CD8+/FoxP3+ after therapy were associated with OS and PFS (progression-free survival) ¹⁹¹. Some studies have even shown this so-called “immunoscore” to outperform the traditional TNM staging in predicting outcomes in CRC ¹⁹², which reveals the impact of host immune response on prognosis.

Protein Expression

Protein biomarkers are the major component of CRC biomarkers. Many researchers consider that proteins are the most accurate biomarkers since proteins are the main executor of human life actions. There are 583 protein biomarkers recorded in the CRC biomarker database.

Expression of proteins including epidermal growth factor receptor (EGFR), vascular endothelial factor (VEGF), p21, Bcl2- Bax, marker of proliferation Ki-67 (ki-67), p53, COX-2, hypoxia-inducible factor 1- α (HIF1- α), thymidylate synthase (TS), E-cadherin, matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2), have been previously associated with response to nCRT ¹⁸⁴. P53 mutations have been described in about 50% of CRC and are associated with an aggressive behavior and resistance to chemoradiotherapy ¹⁹³. Interestingly, Li et al. developed a three-protein biomarker panel consisting of c-MYC, proliferating cell nuclear antigen (PCNA) and tissue inhibitor of metalloproteinases 1 (TIMP1), that together with MRI-detected EMVI improved prediction of prognosis in 329 LARC patients ¹⁹⁴.

Genomic Alterations

Currently, the only genomic variants clinically available to predict patient outcome to therapy (anti-EGFR monoclonal antibodies) is the presence of variants in *KRAS* and *NRAS* (neuroblastoma Ras viral oncogene homolog) in mCRC ¹⁹⁵. In the context of LARC, to date, no genomic alteration can predict patient response or long-term clinical outcomes.

Although most studies of genomics on rectal cancer lack consistency, the presence of variants in *KRAS* has been previously associated with poor response to nCRT and lower probability of pCR ¹⁹⁶. In contrast, numerous studies (including a large retrospective cohort of 1886 patients ¹⁹⁷) showed no difference in response or survival between *KRAS* mutant or wild-type disease ¹⁹⁸.

Variants in *TP53* have been correlated with worse treatment response ¹⁹⁸ and decreased rates of pCR ¹⁹⁹. It has been reported that tumor tissue is enriched with *TP53* variants after chemoradiotherapy ²⁰⁰, which might be explained by an evolutionary advantage of mutant *TP53* created by the administration of therapy.

Also, numerous studies have shown that the presence of variants in both *KRAS* and *TP53* lead to a worse DFS ¹⁹⁸ and decreased likelihood of achieving pCR ²⁰¹. However, larger studies with wider cohort of patients with rectal cancer are needed to determine if this predictive value is real.

A distinct molecular subtype of CRC is characterized by DNA hypermethylation in CpG-rich promoters ²⁰². Studies have validated 20 different methylated regions in rectal cancer patients that have relevance in DFS. No association was still found with response prediction ^{203 204}.

After proteins, RNA is the second leading component of CRC biomarker. The most studied type of RNA studied are the microRNAs and 72 miRNAs have been reported as biomarkers in diagnosis, treatment and prognosis ¹⁸¹. MicroRNA-21 (miR-21) is one of the earliest that have been identified. In 2017, Peng et al. reported that circulating miR-21 was better at diagnosis and tissue miR-21 could be a better prognostic biomarker in CRC. They also stated that the combination with other miRNAs could reach better performance than single miRNA biomarkers ²⁰⁵.

Some studies have also reviewed the association between response to nCRT and SNPs (single nucleotide polymorphisms) in several genes including *TS*, *EGFR*, coiled-coil protein DIX1 (*CCD1*), superoxide dismutase 2 (*SOD2*), IL13, 8-oxoguanine DNA glycosylase (*OGG1*) and methylenetetrahydrofolate reductase (*MTHFR*) ²⁰⁶. The occurrence of SNPs located in miRNA target sites may affect the interaction of miRNA and target genes, and have shown to affect outcome in CRC patients ²⁰⁷.

Microsatellite Instability

Roughly 15% of patients with CRC have dMMR functions, which creates MSI, where intergenic microsatellite is found at highly variable and unregulated lengths in the tumor tissue.

dMMR status, a good prognostic indicator in colon cancer, is rarer (~1-2%) in patients with rectal cancer, thus the impact that it can have on response to chemotherapy agents is not well understood ⁴⁸. Studies have shown a lower pCR rate after nCRT in patients with MSI-high rectal cancer ²⁰⁸ and high levels of MSH6 protein was indicative of improved OS and local control ²⁰⁹. These findings are contradictory to colon cancer outcomes reports ²¹⁰, and also to previous findings about rectal cancer prognosis, in which high MSI improved DFS and OS after nCRT ²¹¹. However, MSI-high patients can be exquisitely sensitive to immunotherapy, with high rates of pCR after neoadjuvant nivolumab and ipilimumab ¹⁵¹.

Tumor mutational burden (TMB) is a quantitative measurement of the frequency of variations in the genome of cancer tissue, quantified using large gene panels or whole exome or genome sequencing. TMB is generally higher in patients with dMMR and MSI-H tumors ²¹².

Gene Expression Profiles and Tumor Heterogeneity Markers

Global gene expression profiling of tumor tissues has the potential to identify gene signatures associated with rectal cancer outcomes. Cancer related nCRT-response genes are involved in pathways of DNA replication, cell cycle and DNA repair, while non-cancer related genes are involved in pathways of drug metabolism ²¹³. Examples of genes that were differently expressed between responders and non-responders were zinc Finger protein 160 (*ZNF160*), X-ray repair cross-complementing protein 3 (*XRCC3*), ATP dependent DNA helicase homolog (*HFM1*), additional sex combs-like protein 2 (*ASXL2*), aldo-keto reductase family 1 member C3 (*AKR1C3*), C-X-C motif chemokine ligand 9 (*CXCL9*), *CXCL10*, *CXCL11*, indoleamine 2,3-dioxygenase 1 (*IDO1*), matrix metalloproteinase-12 (*MMP12*), HLA class II

histocompatibility antigen, DR α chain (*HLA-DRA*) ²¹⁴ and neuronal pentraxin II (*NPT2*) ²¹⁵.

Tumor heterogeneity can be calculated according to the number of variants and median variant allelic frequency (VAF) in a tumor sample. It requires a large sequencing panel (>400 genes) or whole genome sequencing for accurate quantification.

The clonal and subclonal composition of the tumor could provide information regarding response to therapy and prognostic values. In patients with CRC, studies have demonstrated that the identification of resistance-associated variants, whether in small tumor subclones or across all tumor cells, can change time to recurrence after targeted therapies ²¹⁶. Although still in its infancy, large scale metrics such as TMB and heterogeneity indexes might have potential for clinical application.

7.2. Blood-Based Biomarkers

As cancer progresses, cancer cells and stromal products are shed into circulation. The identification of these products in liquid samples (whole blood, blood plasma, urine, cerebral-spinal fluid, ascites or sputum samples) could make them potential “real-time” disease biomarkers for disease monitoring, with regular serial samples. However, circulating biomarkers carry several limitations, such as high levels produced by non-tumor tissue release, and variation according to tumor size and stage ²¹⁷. There are currently very few circulating biomarkers being used in the clinical setting for patients with rectal cancer, which include CEA and CA19-9, discussed above ^{170 176}.

Systemic Inflammatory Ratios

Neutrophil Lymphocyte Ratio (NLR)

Inflammation was reported to promote tumor growth or protect tumor tissue from a host immune response. NLR can be measured in routine differential

white cell counts in the peripheral circulation, and has shown some importance in the prognosis and prediction of response. This marker is inexpensive and can be calculated from laboratory tests routinely ordered in the pre-operative setting.

Theoretically, the development of an immunological biomarker could be used to predict response to immunotherapies. Nevertheless, no clinically biomarkers have yet been validated ²¹⁸. Evidence on the value of NLR in LARC is conflicting. Pretreatment NLR has been shown to be associated with pCR, DFS and OS in several studies (including a meta-analysis of 959 patients), but other publications (including a large cohort of 1500 patients) have not found these associations ²¹⁹.

There is also increasing evidence that radiosensitivity might depend not only on the biological features of tumor cells but also on the tumor microenvironment ²²⁰, reflected on blood count cells in peripheral blood. NLR could be an indicator of response to chemotherapy across mutations, especially for BRAF mutant tumors ²²¹ which are commonly MSI-high ²²². Adversely, NLR was not associated with MMR status ²²³.

Some of the proposed CMS subtypes shown previously present with inflammation. CMS 1 and 3 subgroups, which are characterized by MSI-high, hyper-mutated genotypes, also concur with increased tumor infiltrate ⁵⁶. Thus, in the future, inflammatory indexes could be important for the stratification and decision-making in LARC patients.

In conclusion, NLR seems promising for the clinical setting, as a relatively cheap and simple assay to perform and a part of current routine practice. NLR has already shown, even with relatively low patient number and retrospective design studies, consistent results regarding checkpoint inhibitor response ²²¹. However, further multicentric studies with larger populations are required to confirm these data.

Additional markers

Similar to NLR, the value of other systemic inflammatory markers has not been consistent across studies. Previous studies have reported that low pre-treatment LMRs (lymphocyte-to-monocyte ratio) was associated with worse OS ²²⁴ and DSF ²²⁵. Also, high PLRs (platelet-to-lymphocyte ratio) showed worse response

to therapy ²²⁶. Another study, however, showed no significant association between PLR and survival ²²⁷. Platelets have previously been associated with tumor growth, through angiogenesis and metastatic spread. Likewise, monocytes have been found to induce tumor growth, migration and invasion within the tumor microenvironment ²²⁸.

All these ratios are limited by the highly variable outcomes and cut-off values used across studies, and standardization is needed in subsequent studies. Also, the function of the cellular components of these ratios show some plasticity and may be influenced by interactions with cytokines ²²⁹, which means they can have pro- or anti-tumor effects depending on the environment of cytokines they are exposed to.

The mGPS (modified Glasgow prognostic score) ²³⁰ combines pre-treatment serum levels of CRP (C-reactive protein) and albumin, which are two acute phase proteins, into three different categories of levels. Two systematic reviews ^{230 231} have revealed an association between mGPS and TNM stage, mostly in CRC, and that the association of mGPS with prognosis remained present once adjusted for TNM stage.

Although a growing number of publications have been emerging in this area, small numbers of patients have been studied comparing systemic inflammatory indexes with patient response. Further research with larger samples, correlating these ratios to tumor staging and comparing efficacy with other circulating biomarkers needs to be undertaken.

Circulating tumor DNA

It has been known since 1948, that fragmented DNA circulates in the blood ²³². Circulating tumor DNA (ctDNA) is a tumor derived sub-population of circulating free DNA (cfDNA) in patients with solid tumors. The two hypothesized mechanisms of cfDNA release are passive release through cell death ²³³ and active release via exosomes ²³⁴.

In the passive cfDNA release, apoptosis or necrosis cause the fragmentation of DNA into ~180-200bp (base pairs) fragments ²³⁵. Cell death probably invokes an immune response that releases cfDNA through T-cell mediated cell destruction ²³⁵. Instead, other studies suggested that oncogenic DNA is selectively packaged into

exosomes to promote local or distant oncogenic activity ⁷⁶, or that exosomes are used to secrete harmful DNA in response to DNA damage in tumor cells ²³⁶.

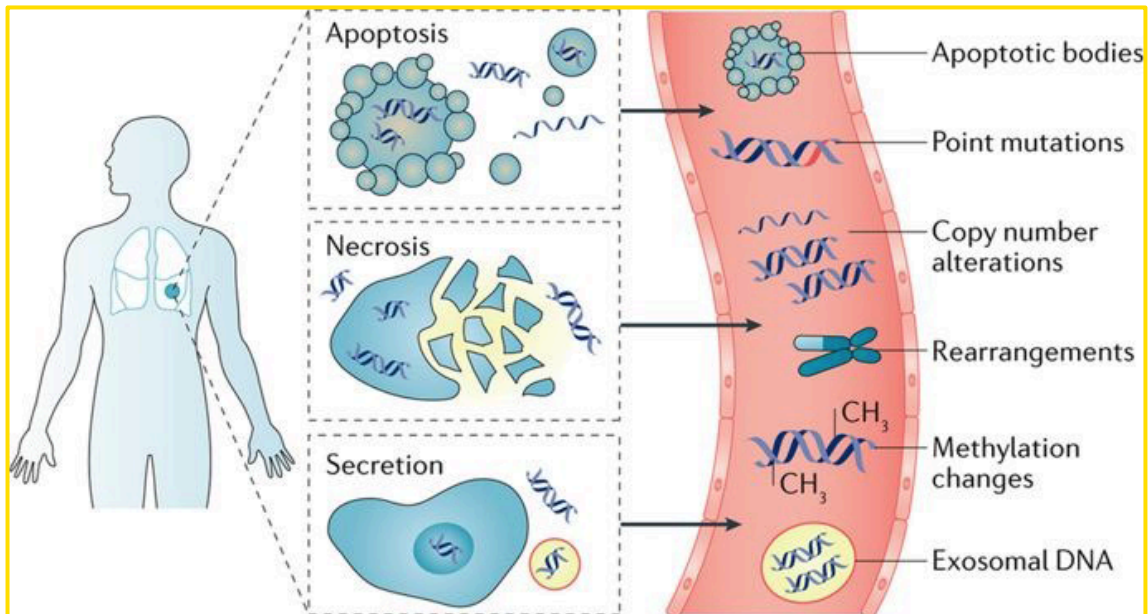


Figure 6. Origins and alterations of ctDNA (reprinted and adapted with permission from ³)

The half-life of cfDNA in blood probably sums up between 16-150 minutes. It may be cleared from the bloodstream and excreted into urine by the kidneys or degraded by macrophages in the liver and spleen ^{3 233}. Plasma cfDNA is highly fragmented and the average reported fragment size revolves around 160bp to 180bp ^{237 238}, and is represented by DNA wrapped around a nucleosome (around 142bp) plus one linker (around 20bp). Some recent evidence suggests that cfDNA and ctDNA differ in size, with ctDNA being shorter than non-mutant DNA, which might result from modifications in chromatin organization and compaction ^{237 239 240}.

Either tumor or non-tumor tissue can release cfDNA into circulation ²³⁵. It has been reported that cancer patients have much higher levels of cfDNA than healthy individuals and that these levels are extremely variable according to tumor burden (tumor stage or the presence of metastases) ²³³. Detection rates range from 50% in non-metastatic disease to nearly 90% in metastatic disease and from 10-15% in postoperative stage II to nearly 50% in stage IV ²¹⁷. Likewise, in rectal cancer, ctDNA levels correlates with tumor size ²⁴¹. We can also distinguish fractions of DNA from both tissues by detecting tumor specific genetic alteration, through methods, such as next-generation sequencing (NGS) or digital polymerase chain reaction (dPCR).

BACKGROUND

Reports suggest that quantitative changes in ctDNA could play a part in therapeutic response in patients with CRC undergoing nCRT. Furthermore, it was reported that ctDNA was a more sensitive method than CEA or radiological imaging in detecting disease progression ²⁴¹⁻²⁴³. CtDNA can circumvent the limitations of tissue biopsies, which include invasiveness, sometimes requiring surgery and the fact that they are only taken from one location, which may not represent the whole heterogeneity of the disease ²⁴⁴. Finally, due to the short turnover time, ctDNA may provide a real-time information about the disease ²⁴⁵.

The analysis of ctDNA for specific gene mutations or somatic copy number alterations (SCNA), such as *KRAS*, *BRAF*, *PIK3CA* and *TP53*, has shown promising results for early detection ²⁴⁶ and response prediction ²⁴¹ in CRC patients. Monitoring disease courses using specific mutations in ctDNA is one of the leading applications of liquid biopsy. In support of this clinical application, some authors demonstrated that concordance of *KRAS* and *BRAF* mutational status between tissue and plasma samples was around 96% ^{247 248}. Post-operative ctDNA VAFs were reported to help predict 3-year DFS after nCRT in patients with LARC ²⁴⁹. At the recent 2022 ASCO (American Society of Clinical Oncology) Gastrointestinal Cancer Symposium, the GALAXY trial confirmed the ctDNA prognostic role in more than 1500 patients with surgically resected CRC (all stages) ²⁵⁰. In patients with metastatic CRC, early decreases in ctDNA indicated improved progression-free survival ²⁴¹, whereas post-surgical ctDNA levels significantly predicted later relapse in patients with CRC undergoing adjuvant therapy ²⁵¹. Also, methylation changes in ctDNA have shown some promising results in CRC management ²⁵².

Presently, though ctDNA most frequently looks to monitor and detect *KRAS/NRAS* mutations, the development of CRC subtyping systems, like CMS and CRIS, will possibly influence the use of ctDNA through alternative mutations, as part of subtype allocation/validation ⁵⁷.

There are several limitations attributed to ctDNA that have to be taken in consideration when investigating patients being treated with cytotoxic agents and surgery, such as the generally low cfDNA concentrations extracted from plasma that can hinder analysis. Besides, a significant amount of cfDNA can originate from non-tumor cells, or induced by inflammation and trauma ²³⁵, which can cause a high background noise and disturb ctDNA detection ²¹⁷ and reliable quantification of

changes in allelic frequency ²³³. Also, ctDNA is often found in short fragments, which can limit the molecular alterations that can be reliably detected. All of these can be aggravated through improper pre-analytical handling ²⁵³.

Analysis of ctDNA is reaching routine clinical use due to its simplicity and relatively low cost. Nowadays, screening of mutations of ctDNA, associated with therapy resistance, is already being applied in non-small cell lung cancer ²⁵⁴. Also kits have been CE-marked for *RAS* screening in ctDNA for patients with CRC ²⁵⁵. These are examples of ctDNA use in the context of targeted therapies with known resistance-associated variants or signatures. Although it still provides relatively limited information, ctDNA is detected in a relatively high proportion of patients and analysis can be screened for mutations of interest, which makes ctDNA a very appealing biomarker. A brief explanation of ctDNA isolation and analysis is presented in Figure 7.

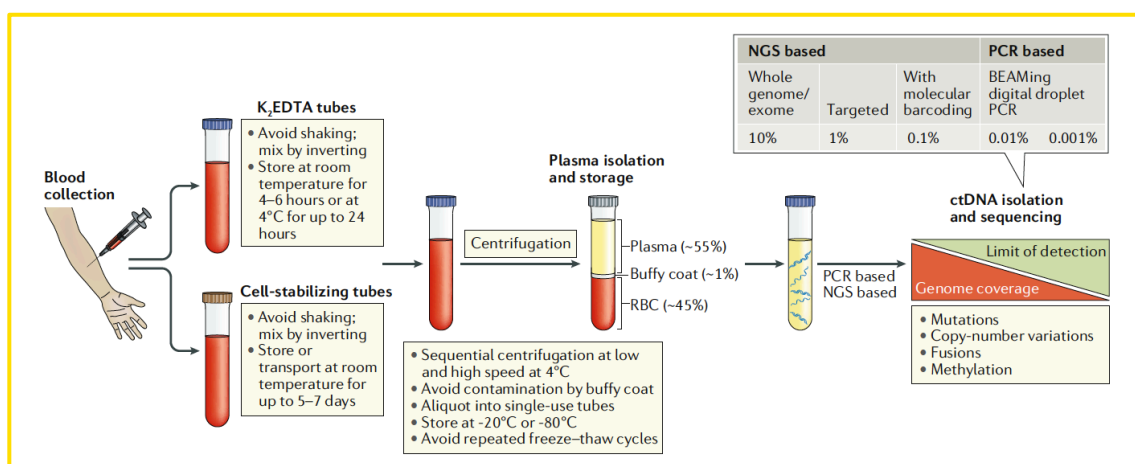


Figure 7. ctDNA isolation and analysis. cfDNA is isolated from plasma samples after serial centrifugation of blood collected in either of the tubes presented. ctDNA is subsequently isolated from cfDNA using library preparations and analyzed for genetic aberrations (reprinted and adapted with permission from ⁵)

In LARC patients undergoing chemoradiotherapy, a variant or signature associated with treatment resistance or sensitivity is yet to be identified. Therefore, ctDNA can hypothetically be used for monitoring and surveillance of disease recurrence, but there is still a long way in the identification of adequate signatures for use in the clinical setting.

Circulating microRNA

MicroRNAs are small non-coding RNAs (~18-25 nucleotides long) that regulate targeted mRNA (messenger RNA) expression in healthy and cancer tissues. They are either located in non-coding intergenic regions of DNA or within gene introns ²⁵⁶. MicroRNAs are relatively stable in circulation due to their protection from RNase activity through their association with carrier proteins or inclusion within exosomes ²⁵⁷.

MicroRNAs have been linked with numerous roles in tumorigenesis, like migration, invasion, metastasis ²⁵⁸, growth, proliferation and inhibition of apoptosis ²⁵⁹. Their function can be either tumor promotor or suppressing, which varies through different cancer types.

Several microRNAs have been studied in patients with CRC for their predictive or prognostic values, although none has yet been validated. Several miRNAs' levels were significantly different among responders and non-responders. The following miRNAs were increased in non-responders: miR-154, miR-409-3p, miR-127-3p, miR-214, miR-299-5p, miR-125b and miR-345; and the subsequent were decreased in non-responders: miR-22a, miR-30e, miR-338-3p, miR-200 and miR-378 ¹⁸⁴.

Although microRNAs do not presently influence subtyping of CRC, the CRCSC has reported that CMS2 tumors up-regulate the miR-17-92 cluster, while CMS3 and CMS4 down-regulate let-7 and miR-200 respectively ⁵⁶. These could potentially be monitored in plasma or plasma exosomes to predict response or stratify patient treatment at some point. While of great interest, they have not yet found clinical application due to lack of consensus over which expression signatures to pursue and their technical limitations.

Circulating Exosomes

Recently, an increasing interest has been shown in the investigation of extracellular vesicles as circulating biomarkers in patients with solid tumors ²⁶⁰. Exosomes are a subset of extracellular vesicles which are ~30-120 nm in diameter.

They have several roles, including cell-to-cell communication ²⁶¹, and have been reported to play key roles in several different aspects of molecular biology, including immune cell modulation, apoptosis and angiogenesis ²⁶². It has been reported that exosomes transport proteins, several RNA species and DNA. These contents may either give an indication of the genetic profile of parent tumor cells or the interaction of tumor cells with surrounding stroma. Certain proteins can also be analyzed to ensure the purity of exosomes isolated ²⁶⁰.

Exosomes increase their levels in the circulation of cancer patients and during disease progression ²⁶³. Some studies have investigated the molecular cargo of circulating exosomes as biomarkers in CRC, with greater emphasis on miRNA. One study reported increased expression of exosome-derived mir-19a as a prognostic biomarker of recurrence in patients with CRC ²⁶⁴. Recently, studies have also investigated tumor-specific alterations in exosome-derived RNA (exoRNA) or DNA (exoDNA) for monitoring patients and stratify therapy, in a similar manner to ctDNA. A study by Hao et al. ²⁶⁵ investigated sequencing concordance, reporting that 14/19 (73.7%) of *KRAS* mutations and 6/8 (75%) of *BRAF* mutations detected in patient tissue were also present in patient exoRNA.

The development of cancer-specific exosomal markers may also facilitate the detection of subtler molecular signatures and limit non-tumor background ²⁶⁶. The goal is to isolate purer tumor-derived exosomes using a specific panel of surface markers and hopefully of CRC-specific exosomes.

The limitations of this marker are the small concentration of exosomes originated purely from the tumor, as previously seen for ctDNA and microRNA, which will likely cause issues in the molecular analysis. The presence of non-tumor exosomes may also mask subtle signatures from tumor-derived exosomes. Hopefully, these issues might be overcome by the development of new technologies ²⁶⁰.

Circulating tumor cells (CTC)

CTCs are cells detected in the circulation of cancer patients which are CD45^{-ve} whilst expressing epithelial markers ²⁵³. The understanding is that CTCs are

BACKGROUND

tumor cells that have been disseminated into peripheral blood and are precursors of distant tumor metastasis ²⁶⁷. To date, CellSearch[®] is the only Food and Drug Administration (FDA) approved method for seizing CTCs in a clinical setting ²⁶⁸, which uses epithelial markers EpCAM (epithelial cellular adhesion molecule) and cytokeratins to isolate and enumerate CTCs.

The potential of CellSearch[®]-based CTC detection was investigated in 11 studies and in a total of 1847 CRC patients ²⁶⁹. They showed that high CTC counts at baseline and throughout treatment were associated with worse PSF and OS. Bork et al. ²⁷⁰ and Tan et al. ²⁷¹ demonstrated the same results in stage I-III and metastatic CRC patients, respectively. Also, some studies suggested that patients that changed from being CTC-high to CTC-low during therapy had better disease control than patients who changed from low to high or remained persistently high ²⁷². In rectal cancer patients, studies have shown that patients with high numbers of CTCs (>5) were more likely to develop metastasis within one year of curative surgery ²⁷³ and patients who responded well to neoadjuvant therapy had lower post-treatment CTCs ²⁷⁴.

Although promising, this biomarker continues to have limitations in rectal cancer patients, due to low specificity. Detection of one or more CTCs per 7.5mL has only been reported in 30-50% of CRC patients, which limits its potential clinical efficacy. Also, a further limitation is the possibility of false negatives due to loss of epithelial markers during epithelial-mesenchymal-transition (EMT) ²⁷⁵. In order to surpass some limitations, new technologies, such as CellCollector[®], are being developed. This technique consists of inserting probes directly into patient blood vessels for an extended period of time in order to collect larger numbers of cells directly from the blood, as opposed to collecting CTCs from a limited volume of whole blood ²⁷⁶.

Furthermore, CTCs have provided additional insight into intra-tumor heterogeneity, as CTCs within a single patient show different mutational profiles. This demonstrates that separate regions of the invasive tumor can have individual driving mechanisms ²⁷⁷. Also, relevant mutations that were absent in tumor biopsies, have been detected in CTCs, influencing patient response ²⁷⁸.

CTCs and ctDNA have often been compared for their potential clinical impact. One study reported a concordance of 78% of CTCs and ctDNA with mutations

detected in primary tumor or metastases ²⁷⁹. Each biomarker was detected in one patient where the other was not and also, each biomarker detected a mutation previously unseen in the primary tumor or metastasis. This fact suggests both biomarkers may be equally useful when considering intra-tumor heterogeneity. One advantage of ctDNA over CTCs is that ctDNA was reported to be detectable in 100% of metastatic CRC, while only 35% had detectable CTC levels from 10mL whole blood ²⁸⁰. Nevertheless, because CTCs detection does not require knowledge of existing tumor-specific changes, it can have a superior role on the screening and early detection.

Circulating metabolites

In LARC patients, CEA levels before and after treatment and changes across treatment were associated with pathological response to nCRT and disease recurrence ^{281 282}. However, CEA has shown limitations as a biomarker in disease monitoring, due to its low sensitivity and specificity ^{283 284}, detecting only 30-60% of recurrences. Likewise, large decreases of CA19-9 concentration were associated with increased rate of pCR ²⁸⁵ while elevated pre-treatment CA19-9 predicted reduced OS ²⁸⁶, although contradictory findings have been found ²⁸⁷. Nevertheless, the combination of these two biomarkers in a pre- or post-treatment setting may increase sensitivity and specificity for prediction of response and survival in patients with rectal cancer ²⁸⁷. Furthermore, a systematic review and meta-analysis demonstrated that low CEA pre-nCRT levels are correlated with higher likelihood of pCR, better DSF and OS, but suggested that the calibration cut-off should be 3ng/mL instead of the standard 5ng/mL ²⁸⁸.

Further metabolites under investigation include proteins, glycoproteins, amino-acids and lipids. In one study, liquid chromatography with mass spectrometry has been used to analyze sequential patient serum samples from 70 patients with CRC. Using five core-metabolites (succinate, N2-dimethylguanosine, adenine, citraconic acid and 1-methylguanosine), this group demonstrated that they could distinguish patients with progressing and stable disease with sensitivity and specificity of 83% and 94%, respectively, which was superior to CEA concentrations

predictions ²⁸⁹. Fibrinogen is another acute-phase protein that in combination with CEA levels was a significant predictor of downstaging, primary tumor regression and pCR after nCRT ²⁹⁰.

The CRCSC has reported a number of metabolic pathways that are deregulated within CMS3 ⁵⁶. This is in agreement with a systematic review published by Zhang et al. ²⁹¹. They reported deregulated levels of amino acids and carbohydrate metabolism in several tissues. Thus, metabolomic analysis of patients' tumor, plasma or urine may become important for patient subtyping into a future uniform system.

In Figure 8, the main biomarkers discussed above, associated with response to nCRT and prognosis, are summarized in a synoptic diagram.

Summary of biomarkers



Figure 8. Synoptic diagram of biomarkers associated with response to nCRT and prognosis in patients with LARC

THESIS OVERVIEW: AIMS AND METHODS



Thesis Overview: Aims and Methods

Organ preserving strategies open a branch of options in the future of locally advanced rectal cancer treatment strategies. Therefore, the search for more specific and readily available circulating biomarkers is a research field in expansion. Also, the possibility to provide better personalized treatment options, such as total neoadjuvant treatment or selective use of adjuvant treatment, according to different prognostic groups, makes circulating biomarkers an attractive option.

This research project aimed to validate circulating markers that could be readily available in liquid biopsy and evaluate its efficacy in predicting response to nCRT and prognosis in LARC patients. We first intended to evaluate the host-response and its impact on outcomes, since most of circulating inflammatory markers are already requested in the clinical setting. Furthermore, with the intent to add more information on circulating biomarkers, we then focused our study on ctDNA, which has shown promising results in the literature. As mentioned before, this biomarker is readily available through liquid biopsy, allows the monitoring of disease in real-time and reflects tumor heterogeneity and dynamic alterations in tumor burden. This makes ctDNA a promising marker for predicting treatment efficacy and disease recurrence.

To achieve this purpose, we performed the following works, to evaluate specific aims.

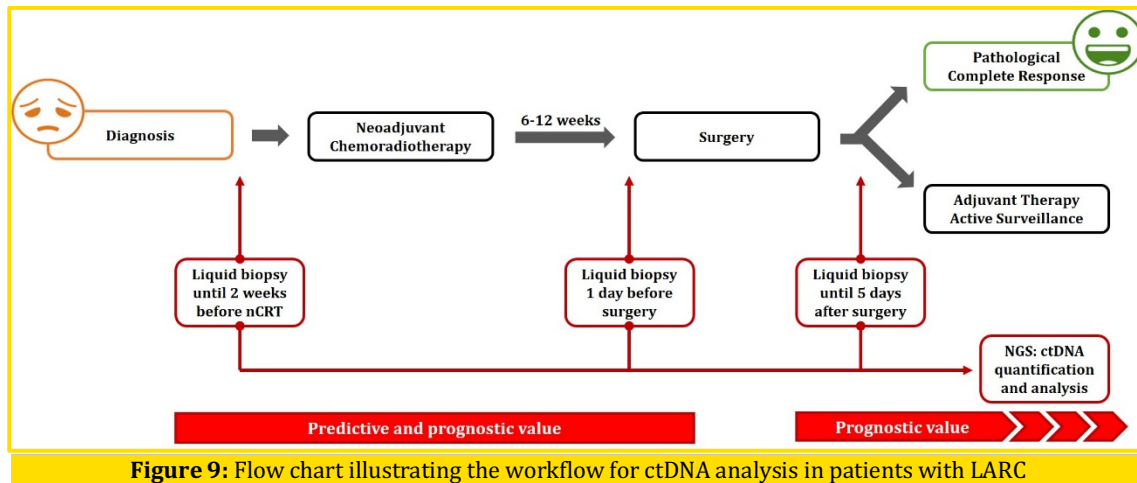
Evaluation of the role of circulating hematological and biochemical biomarkers on the prediction of response to nCRT and prognosis in LARC patients:

- Two-center retrospective study of 10 years to validate the impact of baseline inflammatory markers in predicting response to nCRT. Logistic regression analysis to search for predictive markers of pathological complete and good response.
- Two-center retrospective study of 10 years to validate the impact of baseline and post-treatment circulating markers in predicting prognosis in LARC patients. Cox regression analysis to search for predictors of prognosis. Study

of survival outcomes by Kaplan-Meier method to define subgroups of prognosis.

Evaluation of the role of ctDNA in predicting pathological response to nCRT and prognosis in LARC patients:

- Systematic review of the literature on the role of cfDNA and ctDNA in the prediction of response to nCRT and prognosis in LARC patients;
- Multicentric prospective study (3 centers) of 2 years to validate the impact of baseline and serial ctDNA levels (measured through NGS), in the prediction of response to nCRT and prognosis. For this study, which intended to evaluate the impact of ctDNA in response and prognosis, the workflow exhibited in Figure 9 was proposed in the study protocol and performed as explained in the next chapters. Due to contingences on the budget acquired and due to the low number of recruited patients due to covid pandemic and time span of the study, completion of the full protocol still awaits future funds and more follow-up.



RESULTS



Article 1

Can pretreatment blood biomarkers predict pathologic responses to neo-CRT in patients with LARC?

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Research Article

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Can pretreatment blood biomarkers predict pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer?

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Aims: To investigate the value of previously described pretreatment hematological and biochemical biomarkers as predictors of pathological response. **Methods:** The authors performed a retrospective analysis of 191 patients with locally advanced rectal cancer who underwent long-course neoadjuvant chemoradiotherapy at two Portuguese centers. The authors performed logistic regression analysis to search for predictive markers of pathological complete and good response. **Results:** High platelet–neutrophil index ($p = 0.042$) and clinical tumor stage ≥ 2 ($p = 0.015$) were predictive of poor response. None of the analyzed biomarkers predicted pathological complete response in this study. **Conclusion:** A high platelet–neutrophil index before neoadjuvant chemoradiotherapy could help predict poorer pathological response in patients with locally advanced rectal cancer. However, no other blood biomarker predicted incomplete or poor response in this study.

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Keywords: carcinoembryonic antigen • lymphocyte-to-monocyte ratio • neoadjuvant chemoradiotherapy • neutrophil-to-lymphocyte ratio • pathological response • personalized medicine • platelet–neutrophil index • platelet-to-lymphocyte ratio • predictor • prognostic nutritional index • rectal cancer • systemic immune–inflammation index

Colorectal cancer is the third most commonly diagnosed type of cancer and the second leading cause of cancer death worldwide [1]. There should be utmost concern for this disease, as its global burden is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 [2]. Approximately 40% of colorectal cancer patients can be classified as having rectal cancer.

The standard treatment regimen for locally advanced rectal cancer (LARC) is neoadjuvant chemoradiotherapy (neo-CRT) followed by total mesorectal excision, as this improves tumor downstaging and leads to higher rates of sphincter-sparing surgery and better local control [3–5] while having a lower toxicity profile compared with postoperative chemoradiation [6]. Nevertheless, response to neo-CRT is widely variable between patients. Approximately 75% of patients respond well to neo-CRT and about 10–30% achieve a pathological complete response (pCR) in which no viable tumor cells are present in surgical resection specimens [7]. There is a demonstrated advantage to achieving a clinical/radiographic complete response and pCR in these patients, as studies have shown improved long-term outcomes, including disease-free survival (DFS) and overall survival [8]. Furthermore, given the adverse effects of surgery (bowel, urinary and sexual dysfunction) and possible postoperative complications (anastomotic leakage and wound healing impairment) in patients who achieve pCR after neo-CRT, alternative approaches, such as the ‘watch and wait’ strategy, have been suggested [9]. However, up to 25% of patients exhibit resistance to neo-CRT, displaying either minimal regression or complete lack of response [10,11]. The use of neo-CRT in these patients may result in disease progression or delay in surgery, thus affecting resectability [12]. These patients could be

candidates for alternative neoadjuvant approaches, such as 'total neoadjuvant therapy,' which combines preoperative chemotherapy with neo-CRT [13], or be considered for upfront surgery.

Indeed, the mechanisms underlying the observed heterogeneity of tumor sensitivity to neo-CRT are still not well understood [14]. As the ability to predict response in patients undergoing neo-CRT for LARC could allow for individualized cancer-directed treatment [15], a wide range of clinical, radiological, serological, histopathological and genetic factors have been studied as potential predictors [16–18]. In recent years, blood biomarkers have become attractive as predictors because they are easy to collect, relatively inexpensive, reflect information on different aspects of tumor biology and can be accurately measured by standardized methods.

Traditionally, chemotherapy and radiotherapy destroy tumor cells in a conventional cytotoxic manner, but recent evidence suggests that these treatments could provoke an immune response, which also causes tumor regression [19]. Thus, an important factor contributing to the aforementioned heterogeneity might be the differential interaction between the individual tumor microenvironment and the patient's immune system [20]. This tumor-related inflammatory response is reflected by several hematological parameters. The most studied blood biomarker for response to neo-CRT in rectal cancer is carcinoembryonic antigen [21]. The predictive value of mean corpuscular volume [22], prognostic nutritional index [23] and CRP [24] has also been analyzed. Likewise, it has been reported that several inflammatory indexes, such as the systemic immune-inflammation index (SII), lymphocyte-to-monocyte ratio (LMR), platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), platelet–neutrophil (PN) index and total white blood cell count, might be able to predict the response to neo-CRT in rectal cancer [25–30]. However, results diverge between these studies [31,32], and few have investigated these potentially significant biomarkers together [33]. Therefore, the authors conducted a multi-institutional study to evaluate the value of most of these pretreatment blood biomarkers together in predicting the pathological response to neo-CRT in patients with LARC.

Methods

Subjects

The authors performed a retrospective analysis of consecutive patients who underwent long-course neo-CRT followed by radical surgery for rectal adenocarcinoma at Centro Hospitalar Universitário do São João (Porto, Portugal) and Hospital Pedro Hispano (Matosinhos, Portugal) from January 2011 to December 2020. The ethics committee of each hospital approved the study. The inclusion criteria were pathological diagnosis of adenocarcinoma, no reported metastasis and complete clinical records, including pathological characteristics of the tumor, therapeutic interventions and laboratory data (except for albumin, which was not requested in some patients). The exclusion criteria were primary malignancies of other organs, patients with previous antitumor treatment, clinical evidence of chronic/acute infection or systemic inflammation, hematological diseases, autoimmune diseases and patients previously treated with immunosuppressive therapy.

Data retrieval

Clinicopathological variables retrieved from the electronic medical record system of both hospitals included age, sex, clinical presentation (bowel obstruction, perforation or change in bowel habits, classified as high-risk presentation, and diagnosis with screening or bleeding/anemia, classified as low-risk presentation), tumor length, distance of tumor to anal verge, clinical tumor and node stage, treatment regimen, time interval from the end of neo-CRT to surgery (in weeks), pathological response and hematological or biochemical parameters collected between weeks 1 and 4 prior to neo-CRT.

Treatment

All patients were treated with long-course neo-CRT. The total dose of radiotherapy was 50.4 Gy, and the single dose was 1.8 Gy. The concurrent chemotherapy regimens used included capecitabine, 5-fluorouracil or combinations of one of the two substances with oxaliplatin or leucovorin plus oxaliplatin. No patient in this analysis received induction or consolidation chemotherapy regimens. Surgical resection with total mesorectal excision was planned for the 6th–12th week after the completion of neo-CRT irrespective of the response to neo-CRT.

Pathological evaluation

Postoperative histopathological classification and staging of rectal tumors were reported according to the classification set by the American Joint Committee on Cancer [34]. Pathological response to neoadjuvant therapy was

Can pretreatment blood biomarkers predict pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer? Research Article

categorized into two sets of outcomes. One was pCR (no residual tumor on postoperative histological evaluation of the rectal surgical specimen) versus incomplete response (partial and no pathological response). The second was good versus poor response, which was determined according to the Ryan tumor regression grade (TRG) classification [35] by ascertaining the number of residual tumor cells compared with the desmoplastic response. The Ryan TRG classification was used in this study since it is the classification used by both anatomical pathology departments. The 'good response group' was defined as TRG 1 (no viable cells or single cells or small groups of residual cancer cells), and the 'poor response group' was defined as TRG 2 (residual cancer outgrown by fibrosis) or TRG 3 (significant fibrosis outgrown by cancer or no fibrosis with extensive residual cancer).

Blood parameters & inflammatory scores

The recorded blood parameters at diagnosis were hemoglobin; mean corpuscular volume; white blood cell count; absolute counts of neutrophils, lymphocytes and monocytes; platelets; albumin; and carcinoembryonic antigen. Although CRP is used frequently in pretreatment scores, this parameter is not routinely requested by either institution involved in this study, and it was therefore not assessed. Next, the most used inflammatory scores in the literature were calculated. NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. PLR was calculated by dividing the platelet count by the absolute lymphocyte count. LMR was calculated by dividing the absolute lymphocyte count by the absolute monocyte count. SII was calculated by multiplying the platelet count by the absolute neutrophil count and dividing by the absolute lymphocyte count. The prognostic nutritional index was calculated by adding the albumin count to the multiplication of 5 per the absolute lymphocyte count. The PN index was established by creating high and low strata groups of pretreatment platelet and neutrophil counts. The high PN index group was defined as having at least one pretreatment platelet or neutrophil count above its respective threshold ($350 \times 10^9/l$ for platelets and $7.5 \times 10^9/l$ for neutrophils). The low PN index group was defined as having both pretreatment platelet and neutrophil counts below their respective thresholds.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 26.0 (IBM Corporation, NY, USA) for Mac (Apple, CA, USA). The authors summarized patient demographics and baseline characteristics using descriptive statistics. Draft analysis of individual variables was conducted using chi-square test for categorical variables and Student's *t*-test and Mann-Whitney test for continuous variables on the basis of the presence of a normal distribution. Univariate and multivariate logistic regression analysis was used to determine the significant predictors of pathological response to neo-CRT. Continuous variables were analyzed as continuous or dichotomous variables using the median as the cutoff value. Significant variables in the univariate analysis were included in the multivariate analysis using stepwise backward logistic regression to determine which variables retained independent significance. Odds ratios (ORs) are presented with 95% CIs, and $p < 0.05$ was considered statistically significant. The authors assessed the positive and negative predictive value of the PN index using chi-square analysis. DFS and cancer-specific survival (CSS) were calculated from the date of surgery to the date of disease recurrence and date of death attributed to cancer, respectively. Kaplan-Meier curves of DFS and CSS were constructed, and the log-rank test was employed to compare the following outcomes: pCR versus incomplete response and good versus poor response.

Results

Patient characteristics & baseline parameters

Patient characteristics are shown in Table 1. In total, 191 patients who underwent neo-CRT followed by radical surgery for rectal adenocarcinoma were included in the authors' study. Of these, 152 (79.6%) came from Centro Hospitalar Universitário de São João and 39 (20.4%) came from Hospital Pedro Hispano. There were no significant differences between the patients from Centro Hospitalar Universitário de São João and Hospital Pedro Hispano, except for the interval of time from neo-CRT to surgery (median: 10 vs 7 weeks; $p < 0.001$). A total of 101 (63.4%) of the patients were male and 70 (36.6%) were female. The median age of the authors' cohort was 62 years (range: 28–84 years). Most patients were diagnosed with clinical stage III disease (80.1%). The majority of patients were treated with capecitabine (90.6%) and received a higher than 50-Gy dose of neoadjuvant radiotherapy (99.5%). Altogether, 24 (12.6%) patients achieved pCR and 89 (46.6%) were categorized as good responders.

Table 2 shows the levels of pretreatment blood parameters and biochemical markers in the pCR versus non-pCR and good response versus poor response groups. In general, lower serum levels of mean corpuscular volume, NLR

Table 1. Baseline patient and tumor characteristics.

Variable	Patients, n (%)	pCR			Response		
		Yes	No	p-value	Good	Poor	p-value
Patient characteristics							
Age, years [†]	62 (17)	62 (22)	62.5 (17)	0.992	62 (18)	62 (18)	0.564
Sex				0.001			0.116
Male	121 (63.4)	8	113		51	68	
Female	70 (36.6)	16	54		38	32	
Clinical presentation				0.221			0.385
Low risk	42 (22)	8	113		51	68	
High risk	79 (41.4)	16	54		38	32	
Tumor features							
Tumor length				0.008			0.022
≤5 cm	99 (51.8)	18	74		53	39	
>5 cm	(47.6)	5	78		33	48	
Tumor location				0.275			0.804
≤6 cm	92 (48.2)	15	84		47	51	
>6 cm	83 (43.5)	9	82		42	48	
Clinical T stage				0.019 [‡]			0.013 [‡]
T2	14 (7.3)	5	9		11	3	
T3	138 (72.3)	19	119		62	74	
T4	39 (20.4)	0	39		16	23	
Clinical N stage				0.052			0.104
N (-)	37 (19.4)	1	36		13	24	
N (+)	153 (80.1)	23	130		76	75	
Mucinous histological type	2 (1.0)	0	2	1.000	2	0	0.223
Neoadjuvant treatment							
Chemotherapy regimen				0.791 [‡]			0.698 [‡]
Incomplete	2 (1)	0	2		0	2	
Capecitabine	173 (90.6)	23	150		81	90	
5-fluorouracil	10 (5.2)	0	10		6	4	
Other [‡]	6 (3.1)	1	5		2	4	
Radiation dose >50 Gy	190 (99.5)						
CRT-surgery interval <10 weeks [‡]	118 (61.8)	15	103	0.938	51	67	0.200
Outcome							
pCR	24 (12.6)						
Good response [§]	89 (46.6)						

[†] Continuous variables presented as median (interquartile range).

[‡] Other chemotherapy regimens: XELOX (capecitabine + oxaliplatin) and FOLFOX (leucovorin + 5-fluorouracil + oxaliplatin).

[§] Good pathological response: Ryan TRG 1.

[¶] cT2 versus cT3–T4 and incomplete chemotherapy regimen versus complete.

c: Clinical; CRT: Chemoradiotherapy; N: Node; pCR: Pathological complete response; T: Tumor; TRG: Tumor regression grade.

[†] Continuous variables presented as median (interquartile range).

[‡] Other chemotherapy regimens: XELOX (capecitabine + oxaliplatin) and FOLFOX (leucovorin + 5-fluorouracil + oxaliplatin).

[§] Good pathological response: Ryan TRG 1.

[‡] cT2 versus cT3–T4 and incomplete chemotherapy regimen versus complete.

c: Clinical; CRT: Chemoradiotherapy; N: Node; pCR: Pathological complete response; T: Tumor; TRG: Tumor regression grade.

and PLR were recorded in the non-pCR and poor response groups, whereas lower serum levels of neutrophils, lymphocytes, platelets, carcinoembryonic antigen, SII and PN index were observed in the pCR and good response groups.

Analysis of predictors of non-pCR & poor response

Table 3 shows the predictive factors for non-pCR and poor response to neo-CRT. Variables with no statistical or clinical significance in the draft analysis have been excluded. Univariate logistic regression analysis revealed that male sex, tumor length >5 cm, clinical tumor stage >2 and higher white blood cell and absolute monocyte counts were significant predictors of non-pCR, whereas multivariate logistic regression analysis showed that male sex (OR: 5.288; 95% CI: 1.782–15.692; $p = 0.003$), tumor length >5 cm (OR: 3.488; 95% CI: 1.168–10.423; $p = 0.024$) and clinical tumor stage >2 (OR: 6.188; 95% CI: 1.421–26.948; $p = 0.015$) were significant predictors of non-pCR.

With regard to poor pathological response, univariate analysis indicated that tumor length >5 cm, clinical tumor stage >2 and high PN index were significant predictors. Multivariate logistic regression analysis revealed

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Table 2. Baseline biomarker levels (median and interquartile range) and comparisons between groups.

Variable	Patients, n	pCR			Response		
		Yes	No	p-value	Good	Poor	p-value
Hemoglobin, g/dl	13.3 (2.2)	13.3 (2.2)	13.3 (2.1)	0.641	13.3 (1.8)	13.5 (2.1)	0.713
MCV, fl	88.5 (6.6)	89.6 (9.1)	88.5 (6.2)	0.559	89.5 (6.8)	88.2 (6.3)	0.247
WBCs, 10 ⁹ /l	7.62 (2.88)	6.48 (3.78)	7.90 (2.89)	0.034	7.74 (3.02)	7.66 (2.77)	0.529
Neutrophils, 10 ⁹ /l	4.79 (2.19)	4.05 (1.88)	4.87 (2.19)	0.065	4.77 (2.36)	4.87 (2.24)	0.350
Lymphocytes, 10 ⁹ /l	1.90 (0.80)	1.82 (0.60)	2.00 (0.90)	0.473	1.80 (0.70)	2.00 (0.90)	0.732
Monocytes, 10 ⁹ /l	0.60 (0.33)	0.54 (0.28)	0.62 (0.34)	0.034	0.64 (0.33)	0.58 (0.32)	0.778
Platelets, 10 ⁹ /l	235 (90)	230 (113)	236 (91)	0.200	230 (85)	240 (110)	0.197
Albumin, g/l [†]	41.6 (4.6)	41.9 (5.8)	41.6 (4.3)	0.473	40.7 (4.5)	42.0 (4.2)	0.142
CEA, ng/ml	4.14 (9.70)	2.50 (6.60)	4.45 (9.54)	0.022	3.00 (6.20)	4.69 (12.00)	0.004
NLR	2.45 (1.65)	2.46 (1.25)	2.43 (1.71)	0.348	2.45 (1.45)	2.43 (1.73)	0.498
PLR	128 (75)	142 (64)	127 (79)	0.889	131 (80)	127 (71)	0.539
LMR	3.25 (1.88)	3.58 (1.75)	3.19 (1.95)	0.380	2.90 (1.76)	3.33 (1.99)	0.711
SII	612 (458)	567 (407)	612 (503)	0.149	586 (471)	612 (493)	0.319
PNi	51.0 (5.8)	51.8 (6.1)	51.0 (5.9)	1.000	50.4 (7.3)	51.2 (5.2)	0.218
High PN index	33 (17.3)	1 (4.2)	32 (19.2)	0.084	10 (11.2)	23 (23)	0.036

[†] Total of 40 missing cases (20.9% of the total sample).
 CEA: Carcinoembryonic antigen; LMR: Lymphocyte-to-monocyte ratio; MCV: Mean corpuscular volume; NLR: Neutrophil-to-lymphocyte ratio; pCR: Pathological complete response; PLR: Platelet-to-lymphocyte ratio; PNi: Platelet-neutrophil; PNI: Prognostic nutritional index; SII: Systemic immune-inflammation index; WBCs: White blood cells.

Table 3. Logistic regression analysis of significant predictors of pathological complete and good response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer.

Variable	pCR				Good response			
	Univariate, OR (95% CI)	p-value	Multivariate, OR (95% CI)	p-value	Univariate, OR (95% CI)	p-value	Multivariate, OR (95% CI)	p-value
Age >60	0.711 (0.294–1.715)	0.447			0.926 (0.526–1.665)	1.643		
Sex, male	4.185 (1.687–10.382)	0.002	5.288 (1.782–15.692)	0.003	1.683 (0.874–2.868)	0.130	1.914 (0.988–3.670)	0.051
Tumor length >5 cm	3.795 (1.340–10.742)	0.012	3.488 (1.168–10.423)	0.024	1.977 (1.078–3.624)	0.028	NS	NS
cT >2	4.620 (1.402–15.221)	0.012	6.188 (1.421–26.948)	0.015	4.560 (1.229–16.915)	0.023	6.980 (1.456–33.456)	0.015
cN+	0.157 (0.020–1.202)	0.075	0.222 (0.027–1.815)	0.160	0.535 (0.253–1.128)	0.100	NS	NS
CRT-surgery interval <10 weeks	1.966 (0.399–2.336)	0.938	NS	NS	0.765 (0.403–1.454)	0.414	NS	NS
High PN index					2.360 (1.054–5.283)	0.037	2.510 (1.036–6.081)	0.042
WBCs [†]	1.291 (1.026–1.624)	0.029	NS	NS				
Monocytes [†]	11.226 (1.266–99.511)	0.030	NS	NS				

Clinical presentation, tumor location, pretreatment mucinous histological type, CRT regimen and blood parameters were NS in univariate and multivariate analysis. Scores for hemoglobin, MCV, neutrophils, lymphocytes, platelets, albumin, CEA, NLR, PLR, LMR, SII and PNI (measured as continuous and dichotomous variables using median as cutoff value) are not included.
[†] Measured as continuous variables.
 CEA: Carcinoembryonic antigen; cN: Clinical node; CRT: Chemoradiotherapy; cT: Clinical tumor; LMR: Lymphocyte-to-monocyte ratio; MCV: Mean corpuscular volume; NLR: Neutrophil-to-lymphocyte ratio; NS: Nonsignificant; pCR: Pathological complete response; PLR: Platelet-to-lymphocyte ratio; PNi: Platelet-neutrophil; PNI: Prognostic nutritional index; SII: Systemic immune-inflammation index; WBCs: White blood cells.

that clinical tumor stage >2 (OR: 6.980; 95% CI: 1.456–33.456; $p = 0.015$) and PN index (OR: 2.510; 95% CI: 1.036–6.081; $p = 0.042$) correlated significantly with poor response.

The authors further explored these results and calculated the positive and negative predictive values of PN index in predicting poor response. For the whole sample, PN index had a positive predictive value of 50.6% and a negative predictive value of 69.7% for predicting poor response ($p = 0.033$). In patients with clinical stage III disease, PN index showed a positive predictive value of 48.3% and a negative predictive value of 71.9% ($p = 0.038$).

In the survival analysis, 44 patients died, with a median CSS of 32 months, whereas 121 patients were alive at last follow-up, with a median follow-up time of 54 months. Regarding DFS, 61 patients had progression of disease (13 locoregional and 48 systemic disease) at a median of 17 months, and 130 patients who did not progress had a median follow-up of 49.0 months.

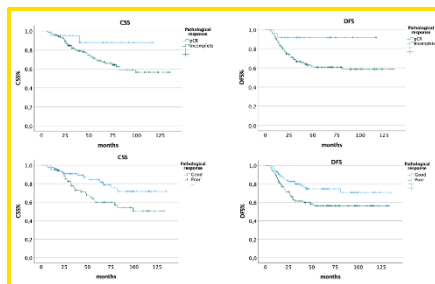


Figure 1. Kaplan–Meier cancer-specific and disease-free survival curves in months for patients with complete and incomplete and good and poor treatment response.

The Kaplan–Meier survival analysis demonstrated no significant differences in DFS or CSS with regard to the PN index groups (log-rank $p = 0.298$ and 0.166 , respectively). The pCR patients had better DFS than incomplete pathological response, but no difference was noted with regard to CSS (log-rank $p = 0.015$ and 0.097 , respectively). DFS and CSS of patients with a good response were significantly better than those of patients with a poor response (log-rank $p = 0.018$ and 0.020 , respectively). These results are shown in Figure 1.

Discussion

The clinical records of 191 patients were retrospectively reviewed and demonstrated that 24 (12.6%) patients achieved pCR and 89 (46.6%) had a good response. The only blood biomarker that predicted poor response was PN index. No other blood marker was able to predict complete pathological response.

The two main outcomes in the authors' study were pCR and good response, both of which have previously been shown to be correlated with better overall survival and DFS in digestive cancer patients [36,37]. Good response was also associated with better CSS and DFS in the authors' series, and pCR was associated with better DFS. A pCR rate of 12.6% is reported in this study, although in some studies it can go up to 38% [38]. This fact can be explained by the more common use of other chemotherapy regimens (5-fluorouracil or the addition of other drugs to capecitabine), administration of induction or consolidation therapy or higher intervals of time between neoadjuvant treatment and surgery than those seen in the authors' series. The identification of patients with poor response could allow us to improve both quality of care and survival by tailoring therapies, such as higher-dose neo-CRT, total neoadjuvant therapy and longer intervals between neo-CRT and surgery, in specific patients [39,40]. A recent meta-analysis of predictive factors for pathological response to neoadjuvant chemoradiation in rectal cancer established that there is currently a lack of robust clinical or radiological biomarkers for such a response [41]. Molecular profiling could be a promising tool in the future, but this is currently unavailable in most practices. Accurate blood biomarkers might be an inexpensive and pragmatic approach to treatment response prediction.

An increased inflammatory response is closely associated with both progression of tumors and poor prognosis in numerous cancers [42]. A plethora of inflammatory scores, including SII, LMR, PLR and NLR, have been shown to be independent prognostic factors in rectal cancer patients treated with neo-CRT [27,28,43]. With regard to their role in predicting response to neo-CRT, a recent systematic review and meta-analysis demonstrated an increased likelihood of pCR in patients with low NLR [44], whereas another study revealed elevated PLR to be a significant predictor of poor pathological response in rectal cancer patients following neo-CRT [45]. However, only a few authors have studied several of these markers simultaneously [33,43]. Li *et al.* reported that low pretreatment LMR and high NLR and PLR were related to poor response after neo-CRT in patients with LARC [33]. However, Kim *et al.* found that pretreatment NLR, PLR and LMR were unable to distinguish total regression from residual disease after neo-CRT [45]. These results demonstrate that data correlation between tumor responsiveness and inflammatory markers is limited and inconsistent [29,43,46].

The results of the authors' study similarly showed that most of the previously described scores, including NLR, PLR, LMR, SII and prognostic nutritional index, were unable to predict responsiveness to neo-CRT in LARC patients. This could be due to the small size of the sample or other confounding factors, but the authors also need to point out the potential impact of publication bias favoring positive results in other studies of this nature. In the authors' study, only high pretreatment PN index was associated with poorer pathological response after neo-CRT,

mainly in patients with clinical stage III disease. Similar to the literature reporting pathological stage as a main prognostic factor and treatment response determinant, the authors found clinical tumor stage to be significant for treatment response. Patients diagnosed with earlier tumor stage disease were more likely to respond completely to treatment than those diagnosed with advanced disease ($p = 0.015$). In clinical practice, a patient with pretreatment platelet count higher than $350 \times 10^9/l$ or neutrophil count higher than $7.5 \times 10^9/l$ could thus be considered for more aggressive neoadjuvant approaches, as described earlier, although more studies are needed to confirm this result. A study by Policicchio *et al.* is in agreement with the authors' study, in that PN index at the time of diagnosis had predictive value ($p = 0.039$) in 112 patients with respect to complete pathological response to neoadjuvant treatment for LARC [30]. In the authors' study, PN index showed reasonable predictive values for poor response to neo-CRT, with a higher negative predictive value, implying that it could be more important for excluding disease and therefore predict good responders. Hence, PN index could be a useful marker in the future for selecting patients for 'watch and wait' approaches, although its value in evaluating pCR could not be tested in this study because of the low rate of pCR, and larger randomized studies are needed to validate these results.

Several mechanisms relate neutrophils and platelets to cancer promotion. Neutrophils produce a nonspecific inflammatory environment in tumors that favors protumorigenic signaling pathways [47] and are also the source of networks of neutrophil-derived DNA expelled in the circulation. Although neutrophils have a physiological role in fighting microbial infections, they have also been observed in some cancers and may be associated with worse prognosis [48]. Several studies also associate neutrophilia with more aggressive and more advanced cancers [49].

With regard to platelets, when entering the tumor microenvironment, they may exchange information with tumor cells and become so-called tumor-educated platelets. Tumor-educated platelets have been observed in the blood of patients with several different types of cancer, and DNA derived from them can be used to discriminate between patients with cancer and healthy controls with high accuracy [50]. Platelets have also been shown to induce changes that result in a loss of adhesion proteins such as E-cadherin [51]. A third mechanism relates to the fact that platelets contain alpha and dense granules that secrete growth factors such as TGF- β , EGF, PDGF, HGF, VEGF, PF-4 and IL-6. Some of these factors contribute to activating signaling pathways that promote malignancy, and some exert effects that promote angiogenesis. IL-6 plays an additional role in inducing liver thrombopoietin [52], which promotes the thrombocytosis often associated with solid cancers.

An interesting finding of this study is the fact that neutrophils and platelets had no individual impact on chemotherapy response, whereas their simultaneous elevation in the bloodstream predicted poorer response. The authors hypothesize that there might be an interaction between neutrophils and platelets that plays a role in the response to neoadjuvant treatment. Neutrophil extracellular traps (NETs), a product of this interaction, were described a decade ago. NETs are formed when activated neutrophils externalize their chromatin and granular content and form a meshwork of DNA strands. Platelets have been found to play an essential role as inducers of intravascular NETs in response to lipopolysaccharides. Conversely, NETs provide a strong activation signal for platelets as a result of externalized DNA and associated histones, promoting platelet aggregation and thrombosis [53]. NETs are initiated and maintained by the action of several binding proteins, including the binding of platelet P-selectin to neutrophil P-selectin glycoprotein ligand-1 and the binding of platelet glycoprotein Ib α to MAC-1 [54].

NETs have been described as directly contributing to malignant progression by sequestration of tumor cells in the circulation of mice with cancer and therefore have been shown to promote metastasis. They have also been detected in individuals with cancer. Agents blocking the formation of NETs or promoting NET dissolution could play a future role in the treatment of these patients [53]. However, the authors found no studies on the potential role of NETs in cancer response to chemotherapy; hence, future studies should begin addressing this issue.

Additionally, some tumor tissue factors were found in the literature to be important factors in predicting the response to neoadjuvant therapy in rectal cancer. Studies have shown mucinous adenocarcinoma to be an independent clinical predictor of not achieving pCR [55]. Although the authors found 13 (6.8%) mucinous adenocarcinomas in the final histology of this analysis, only two were diagnosed preoperatively, and this factor did not stand out in this study as an independent factor for predicting incomplete response. This could be due to small or unrepresentative pretreatment biopsy sampling or possibly to lack of reporting. Moghani *et al.* showed a strong correlation between pathological stage and large numbers of infiltrating lymphocytes and pCR as well as perineural invasion and lymphovascular invasion and poor response [56]. Because of its retrospective nature, and similar to most of the studies evaluating pretreatment blood biomarkers as predictors of response to neo-CRT in LARC, some factors were not taken into account in this study. Although tumor tissue samples might not be representative of the entire tumor tissue, these factors should be taken into account in future studies.

The strengths of this study are that it was conducted at two institutions and included only patients with complete medical records. In addition, most of the important blood biomarkers and scores described in the literature were studied. One limitation of this study is its retrospective nature, which introduces the potential for unmeasured biases. This analysis incorporated a relatively small cohort from only two centers and the rate of one of the outcomes (pCR) was fairly low, so a larger sample is required to make the predictor analysis more reliable; therefore, these results have to be interpreted with caution and cannot be generalized. Another limitation is the fact that, except for mucinous types, histopathological parameters of the tumor biopsy sample were not incorporated into the analysis. A larger, multi-institutional, prospective validation of these results is needed to fully determine the utility of routine blood parameters as predictive factors of response to neo-CRT in LARC.

Conclusion

A high pretreatment PN index might help to predict poor response after neo-CRT in rectal cancer patients, whereas other hematological scores do not seem useful in predicting outcomes. Validation of these results in larger prospective patient populations and further research on inexpensive and practical biomarkers could help personalize the management of patients with LARC.

Summary points

- In recent years, blood biomarkers have become attractive as predictors of pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer (LARC).
- These markers are easy to collect, relatively inexpensive, reflect information on different aspects of tumor biology and can be accurately measured by standardized methods.
- The predictive value of blood biomarkers remains inconsistent across studies.
- The authors performed an analysis of 191 patients with LARC who underwent long-course neoadjuvant chemoradiotherapy and evaluated for predictors of pathological complete and good response.
- The following biomarkers were studied: carcinoembryonic antigen, mean corpuscular volume, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, lymphocyte-to-monocyte ratio, systemic immune-inflammation index, prognostic nutritional index and platelet-neutrophil (PN) index.
- A high PN index ($p = 0.042$) and clinical tumor stage ≥ 2 ($p = 0.015$) were predictors of poor response. None of the analyzed biomarkers predicted pathological complete response in this study.
- PN index predicts poor response reasonably well, mainly in clinical stage III disease.
- A high PN index prior to neoadjuvant chemoradiotherapy could help predict poorer pathological response in patients with LARC, whereas no other blood biomarker was shown in this study to predict incomplete or poor response.
- Validation of these results in larger prospective patient populations is warranted to help personalize the management of patients with LARC.

Author contributions

M Morais and T Fonseca participated in data collection. R Machado-Neves, M Honavar, AR Coelho and J Lopes reviewed the histopathological results. M Morais analyzed the data and was the major contributor in preparing the manuscript. All authors reviewed and approved the final manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Can pretreatment blood biomarkers predict pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer? Research Article

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Can pretreatment blood biomarkers predict pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer? Research Article

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Article 2

Prognostic value of neutrophil-to-lymphocyte ratio (NLR) and platelet-neutrophil (PN) index in locally advanced rectal cancer patients: a retrospective cohort study

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Prognostic value of neutrophil-to-lymphocyte ratio (NLR) and platelet-neutrophil (PN) index in locally advanced rectal cancer patients: a retrospective cohort study

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Abstract

Introduction: In locally advanced rectal cancers (LARC), TNM staging is far from optimal. We aimed to investigate the value of previously described circulating biomarkers as predictors of prognosis.

Methods: Retrospective analysis of 245 LARC patients diagnosed between January 2010 and December 2022, who underwent neoadjuvant chemoradiotherapy and surgery at two centers. A Cox regression and Kaplan-Meier analysis were performed.

Results: Posttreatment platelet-to-lymphocyte ratio (PLR) predicted pCR. The neutrophil-to-lymphocyte ratio (NLR) at two timepoints of the treatment significantly predicted overall survival, whereas the platelet-neutrophil (PN) index significantly predicted disease-free survival. In pathological stage II, the PN index predicted patients with a higher risk of disease-free survival.

Conclusion: Blood parameters might allow the definition of risk subgroups, beyond TNM, for the application of different therapeutic strategies.

Keywords

neoadjuvant chemoradiotherapy, neutrophil-to-lymphocyte ratio, pathological response, personalized medicine, platelet-neutrophil index, platelet-to-lymphocyte ratio, prognosis, rectal cancer

Introduction

Colorectal cancer (CRC) is one of the most common and deadly cancers worldwide ¹. Nowadays, the standard treatment for locally advanced rectal cancer (LARC) is neoadjuvant chemoradiotherapy (nCRT) and radical surgery including total mesorectal excision ². Nevertheless, due to the divergence of treatment response in LARC patients, systemic recurrence remains the main cause of treatment failure ³. Thus, it is crucial to improve the prediction of tumor recurrence, stratification, and prognostication of these patients.

The tumor node metastasis (TNM) staging is accepted as the most powerful prognostic indicator ⁴. Nevertheless, TNM staging is not completely accurate, as patients with the same stage tumor may present different clinical outcomes ⁵. Therefore, other parameters have been considered for subtyping tumors, for example, those included in the Consensus Molecular Subgroups ⁶, such as microsatellite instability, hypermethylation, immune cell infiltrates, immune checkpoints, expression of certain gene pathways and metabolic dysregulation or expression of factors derived from mesenchymal-regulating angiogenesis and stromal invasion.

Inflammation is considered to be involved in tumor progression ⁷ and increasing evidence has shown the correlation of blood inflammatory parameters with worse prognosis of cancers ⁸. Blood circulating biomarkers have become appealing predictors of clinical outcomes, because they are relatively inexpensive and reflect information on several aspects of tumor biology. Either the solitary or combined use of neutrophils, lymphocytes, monocytes, platelets, C-reactive protein (CRP), albumin, and mean corpuscular volume (MCV) have been associated with CRC survival ⁹⁻¹⁷. However, the prognostic implication of combined ratios or scores has not been extensively evaluated in patients with LARC treated with nCRT and there is a heterogeneity of results among multiple studies.

Therefore, this study aimed to investigate the clinical significance of several blood parameter scores, either at baseline or after nCRT, as prognostic predictors in patients with LARC.

Methods

Patients

This cohort study was a retrospective analysis of consecutive rectal cancer patients diagnosed in two hospitals between January 2010 and December 2022. This study was performed in line with STROCSS criteria ¹⁸. Inclusion criteria comprised patients diagnosed pathologically with rectal adenocarcinomas, located within 12cm from the anal verge (measured by MRI), clinically staged T3-4 and/or N+ and treated with neoadjuvant chemoradiotherapy (nCRT) followed by radical surgery. Exclusion criteria were evidence of distant metastasis at diagnosis, emergent or palliative surgery, local excision or “watch and wait” approach and evidence of inflammatory or hematological disorders. The study was approved by the Ethics Committees of both hospitals.

Treatment and Pathologic evaluation

The patients were clinically staged by magnetic resonance imaging. Patients received either one of two neoadjuvant treatment regimens, according to multidisciplinary team decision, taking into account patient and tumor features, and point in time of treatment proposal. The standard neoadjuvant chemoradiotherapy consisted of the administration of a dose of 50.4 Gy of radiotherapy concurrent with capecitabine or 5-fluorouracil (5-FU). The most recent treatment consisted of short-course radiotherapy, with 25 Gy administered over 1 week and followed by neoadjuvant chemotherapy with combinations of 5-fluorouracil and folinic acid or capecitabine, plus oxaliplatin (TNT).

Surgery with total mesorectal excision was scheduled for 6th–12th weeks after completion of nCRT. Adjuvant chemotherapy was recommended selectively to patients for a period of 4-6 months.

The histopathologic examination was performed according to the classification set by the American Joint Committee on Cancer ¹⁹. pCR (pathological complete response) was defined as the absence of any tumor cells at the microscopic examination of the resected specimen on the final pathology ²⁰. All clinicopathological data were retrieved from medical records and summarized in tables.

Follow-up

The frequency of regular outpatient consultations was once every 3 months within 2 years after curative surgery and once every 6 months in the third to fifth years. The follow-up included regular blood

tests, CEA (carcinoembryonic antigen), CA19-9 (carbohydrate antigen 19-9), and chest and abdominopelvic computerized tomography. Also, a colonoscopy was recommended for patients in the first, third, and fifth years of follow-up.

Blood markers and inflammatory scores

Pretreatment blood samples were obtained within 2 weeks before nCRT and posttreatment were obtained before surgery. The blood parameters recorded were hemoglobin, MCV, white blood cell count, absolute counts of neutrophils, lymphocytes and monocytes, platelets, albumin, and CEA.

Next, the most used inflammatory scores in the literature were calculated.

NLR (neutrophil-to-lymphocyte ratio) = neutrophil count / lymphocyte count

PLR (platelet-to-lymphocyte ratio) = platelet count / lymphocyte count

LMR (lymphocyte-to-monocyte ratio) = lymphocyte count / monocyte count

SII (systemic immune-inflammation index) = platelet count x neutrophil count / lymphocyte count

PN (platelet/neutrophil) index: high and low strata groups of pretreatment platelet and neutrophil counts. The high PN index group was defined as having at least one pretreatment platelet or neutrophil count above its respective threshold ($350 \times 10^9/L$ for platelets and $7.5 \times 10^9/L$ for neutrophils).

Receiver operating characteristic (ROC) curve analysis was performed to analyze the area under the curve (AUC) and assess the scores' optimal cut-off values.

Statistical analysis

The data were analyzed using SPSS 26.0 software (IBM Corporation, NY, USA). Clinicopathological characteristics between groups were primarily evaluated using the *Chi-square* test, Student *t*-test, paired samples *t*-test, or Mann-Whitney *U* test, where appropriate. Logistic regression was performed to evaluate predictors of pCR. Overall survival (OS) was calculated as the time from diagnosis to death or last follow-up. Disease-free survival (DFS) was defined as the time from diagnosis until tumor recurrence or last follow-up. Univariable and multivariable analyses (Cox regression model) were used to assess risk factors of the outcomes. Survival outcomes were analyzed by the Kaplan-Meier method and comparisons were assessed by the log-rank test. A two-sided $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 245 patients were included in our analysis, 200 (81.6%) from one center and 45 (18.4%) from another. There were few significant differences between the clinicopathologic features of patients from both hospitals, except for the Charlson score, clinical T stage and interval of time between neoadjuvant treatment and surgery. The clinicopathological features of all patients are shown in Table 1. There were 151 (61.6%) males and 94 (38.4%) females, with 61 ± 12 years on average. Most patients had clinical stage III disease (80.4%) and the majority received nCRT (89.4%), with only 8.2% receiving TNT regimen. Most patients had a stoma performed (91.8%). Of the only 19 patients submitted to anterior rectal resection without a stoma, only 4 presented a leak. The pCR rate was 14.7%. There were 42 missing cases for circumferential rectal margin, 22 for distal rectal margin and mesorectal complete excision was only available in 48.9% of the patients, and therefore we decided to not include this variable in the Cox regression analysis.

The medians (range) of blood markers values are also presented in Table 1. Post-treatment CEA was not available in most patients, so we decided not to include it. Forty-two (18.4%) patients and 32 (13.1%) had a high PN index pre and posttreatment, respectively. The median pretreatment NLR, PLR, LMR and SII levels were 2.47 (0.68-10.79), 128.0 (16.5-801.22), 3.33 (0.40-14.14) and 612 (102.5-7090.8), respectively. The median posttreatment NLR, PLR, LMR and SII levels were 4.50 (0.39-56.67), 257.1 (27.4-969), 1.73 (0.34-9.42) and 926 (98.2-20456), respectively. A paired samples t-test was used to evaluate the difference between means of pre and posttreatment blood markers and scores and in all the difference in means was statistically significant.

Predictive and prognostic value of circulating blood parameters

A logistic regression was performed to evaluate predictors of pCR, shown in Table 2. On multivariable analysis, male gender (OR 4.731, $p < 0.001$) and higher tumor length (OR 1.402; $p = 0.010$) were predictors of incomplete response. Furthermore, post-nCRT PLR also appeared as an independent factor of non-pCR (OR 1.005 (10.001-1.009, $p = 0.008$)). We performed a ROC curve for its value on pCR

and obtained an AUC of 0.616, $p=0.033$, with a value of PLR of 230 corresponding to a sensitivity of 60% and specificity of 57.6% (Figure 1).

The median follow-up was 4 years (ranging from 0-11). The 3-year OS was 81.7% and DFS was 69.5%. These lowered to 69.9% and 65.6% at 5 years mark, respectively. Fourteen patients (5.7%) presented loco-regional recurrence and 63 patients (25.7%) recurred with distant metastasis. According to pathological response, 5-year OS was 81.0% for pCR compared to 68.3% for non-pCR patients ($p=0.080$), and 5-year DFS was 91.7% and 61.6%, respectively ($p=0.004$).

Table 3 shows the analysis for predictors of prognosis. Multivariable analysis revealed that greater longitudinal dimensions of the tumor, pathological stage III, vascular invasion, absence of adjuvant chemotherapy higher and pre-nCRT CEA were significantly associated with mortality. Furthermore, higher pre- and post-nCRT NLR led to a worse prognosis.

As for DFS, greater tumor dimensions, APR patients, pathological stage III, perineural invasion, and higher CRM were associated with more recurrences. Also, higher PN index was an independent factor of recurrence, mainly for loco-regional recurrence (HR 6.909, $p<0.018$), as no statistical significance was achieved for distance metastasis. The analysis of other circulating parameters and prevalent scores in literature, showed that they were not independent predictive factors of prognosis in this analysis.

Finally, a Kaplan-Meier survival analysis was performed to study the impact of the factors found to be significant predictors of survival in the previous analysis, in subtyping LARC patients. These were studied by ROC curve analysis, to find the most accurate cut-off value for prediction of these outcomes. A high pre-nCRT NLR value was considered above 2.5 and a high post-nCRT NLR value above 4 for OS. Patients with affected resection margins (30 patients, 12.2%) were excluded from this analysis. Although results for pre-nCRT were non-significant, we found subgroups of different prognoses for post-nCRT NLR ($p=0.041$). Moreover, the post-nCRT PN index was able to differentiate two subgroups with different disease-free survival ($p=0.014$), even inside pathological stage II ($p<0.001$) (Figure 2).

Discussion

In this study, we found that pre- and post-nCRT NLR were independent factors of OS and that post-treatment PN index was an independent factor of DFS in LARC patients. This was one of the few studies to investigate and evidence the value of several scores at different timepoints in the prognosis of LARC patients. However, other parameters well described in the literature, such as PLR, LMR and SII could not achieve significance in this study. Also, pCR was previously correlated with better prognosis²¹. In our analysis, although not an independent factor, it has shown some impact on survival. A lower post-nCRT PLR was independently associated with pCR, although with moderate accuracy. This association has been observed previously: one study²² has shown that high pre-nCRT platelet levels, changes in platelet levels and post-nCRT PLR were associated with worse pathological response, whereas another report²³ showed that high intra-nCRT and high PLR change rate were predictive of pCR.

The host systemic response plays a significant role in cancer development and progression⁷. Here, we have found an association between worse OS and NLR, an integrated index based on peripheral neutrophil and lymphocytes. The value of NLR in the prognosis of rectal cancer has been extensively described previously²⁴, although these results should be read with caution, since a large-scale study of 1237 patients could not confirm the prognostic or predictive value of NLR or PLR²⁵. Nevertheless, in our study, this score performed better than other scores described in the literature, such as PLR, LMR and SII²⁶. Regarding disease-free survival, post-nCRT PN index, a composite index of platelets and neutrophils, proved to have an impact on our study. These scores allowed the definition of subgroups of risk as shown by the Kaplan-Meier method. Also, inside pathological stage II, in which treatment is still controversial, we could here define further subgroups of disease-free survival, using the PN index. Patients in pathological stage II were proposed to adjuvant CT mainly taking into account pT stage and co-morbidities (Charlson score). The definition of subgroups of prognosis in these patients could help individualize treatment and potentially alter their strategy.

Neutrophils secrete cytokines and chemokines that play an important role in cancer progression²⁷ and neutrophilia have been shown to predict inferior outcomes in rectal cancer²⁸. Lymphopenia is also commonly present in these tumors and might help tumor cells escape immune surveillance and prevent damage by cytotoxic T cells²⁹. Thus, a high NLR level reflects alterations in the cancer microenvironment that favor cancer initiation and progression. High NLR has been previously associated with a worse

response to nCRT^{30,31}, which could help explain why it might be associated with a worse prognosis. Platelets are thought to be involved in tumor progression by regulating neo-angiogenesis and by aiding circulating tumor cells to escape immune surveillance^{32,33}. Several meta-analyses have confirmed that a high NLR and high PLR are significant prognostic factors for poor outcomes in LARC^{34,35}. PN index, an index of neutrophils and platelets, present in the previous scores mentioned, could also have an impact in the prediction of DFS, although this association has not been reported before and deserves further consideration.

To improve DFS and OS and optimize the treatment of patients with LARC, new approaches to treatment and staging are emerging. Several trials studied “total neoadjuvant therapy” (TNT), in which systemic induction chemotherapy precedes nCRT and surgical resection. In the recent RAPIDO trial³⁶, the experimental TNT group increased the rate of pCR and decreased the rate of distant metastases, compared to the conventional approach. On the other hand, some studies have shown that an “immunoscore”³⁷, calculated with counts of CD8+ or FoxP3+ regulatory T-cells, outperformed the traditional TNM staging in predicting outcomes in CCR³⁸ and a plethora of immune checkpoint inhibitors and other immunotherapies are emerging³⁹. Therefore, it seems relevant to assess the impact of inflammatory markers in the subtyping of disease, to select the best strategy for each patient.

There are some limitations to our research. This study was a retrospective analysis with a relatively small sample that included two different neoadjuvant chemoradiation regimens. However, two hospital populations are represented and the results of blood parameters collected in 2 timepoints are presented. In addition, we report that patients with inflammatory or hematological conditions were excluded, but patients with other chronic diseases (such as cardiomyopathy, heart diseases, kidney disease and peripheral arterial disease) were included in order to maintain a substantial sample, although we understand these pathologies could affect the inflammatory condition of the patient and be a confounding variable. Still, the Charlson score, composed by these comorbidities, was analyzed in this study and did not have an independent impact on the outcomes. Also, CRP, albumin and post-nCRT CEA, which has shown prognostic implications in literature, were not routinely examined in both centers and there is a lack of clearly defined cut-off value for inflammatory markers in the literature. Nevertheless, our study adds to the understanding of the prognostic implication of circulating blood parameters in patients with LARC after nCRT, due its long follow-up and evaluation of several baseline parameters. Patients with high NLR in two timepoints and

RESULTS

post treatment PN index might correspond to higher risk groups of stratification and could be proposed for a closer follow-up and more aggressive treatment than those with lower values.

Conclusion

Measurement of circulating parameters in different timepoints across neoadjuvant treatment might enable tailored strategies and prognostication, and consequently improve survival outcomes in LARC patients. However, these results require further validation in larger prospective populations.

Provenance and peer review

Not commissioned, externally peer-reviewed.

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Table 1. Clinicopathological features

Parameters	Values	Parameters	Values
Patient characteristics		Histopathological features	
Gender		Histological stage	
Male	151 (61.6%)	II	179 (73.1%)
Female	94 (38.4%)	III	66 (26.9%)
Age, years	61 ± 12	Grade	
Charlson score		1	20 (8.2%)
0-1	116 (47.3%)	2	157 (64.1%)
≥ 2	129 (52.7%)	3	2 (0.8%)
Body mass index		Vascular invasion	72 (29.4%)
<25	100 (40.8%)	Perineural invasion	59 (24.1%)
≥ 25	126 (51.4%)	Margins	
Tumor features		R 0	214 (87.3%)
Dimensions (longitudinal)		R 1-2	30 (12.2%)
< 5cm	129 (52.7%)	CRM, cm	1 (0-10)
≥ 5cm	99 (40.4%)	DRM, cm	3,5 (0-33,5)
Clinical stage		Mesorectal complete excision	59 (24.1%)
II	48 (19.6%)	Pathologic regression grade (Ryan ⁴⁰)	
III	196 (80.4%)	1	107 (43.7%)
Treatment		2-3	125 (51.1%)
Neoadjuvant chemotherapy		pCR	26 (14.7%)
Capecitabine / 5-FU	219 (89.4%)	Baseline and post-nCRT blood parameters	
TNT	20 (8.2%)	CEA (ng/dL)	4.17 (0.50-358)
Interval neoadjuvant treatment to surgery, weeks	10 (3-51)	Hemoglobin (g/dL)	13.5 (7.3-17.3) → 12.7 (7.9-16.2)
Surgery		MCV (fL)	89.3 (64-110) → 91.9 (43-106)
Anterior resection with stoma	167 (68.2%)	Leucocytes (x10 ⁹ /L)	7.88 (3.2-18.5) → 5.52 (1.95-26)
Abdomino-perineal amputation	148 (88.6%)	Neutrophils (x10 ⁹ /L)	4.92 (1.80-11.9) → 3.9 (0.5-25)
Abdomino-perineal amputation	78 (31.4%)	Lymphocytes (x10 ⁹ /L)	2.00 (0.40-5.80) → 0.83 (0.3-9)
Approach		Monocytes (x10 ⁹ /L)	0.60 (0.21-1.44) → 0.5 (0.18-5.8)
Open	112 (45.7%)	Platelets (x10 ⁹ /L)	243 (66-719) → 212 (40-632)
Laparoscopic	133 (54.3%)	Outcomes	
Total postoperative complications	58 (23.7%)	Follow-up, years	4 (0-11)
Intra-abdominal complications (leaks, abscesses)	25 (10.2%)	3-year OS	81.7%
Adjuvant chemotherapy	142 (58.0%)	3-year DFS	69.5%
		5-year OS	69.9%
		5-year DFS	65.6%
		Loco-regional recurrence	14 (5.7%)
		Systemic recurrence	63 (25.7%)

Values are presented as *n* (%), mean ± s.d. or median (range). BMI, body mass index; CRM, circumferential resection margin; DRM, distal resection margin; pCR, complete pathological response; nCRT, neoadjuvant chemoradiotherapy; CEA, carcinoembryonic antigen; MCV, mean corpuscular volume; OS, overall survival; DFS, disease-free survival.

Table 2. Logistic regression analysis of significant predictors of pCR to nCRT in LARC

Variable	pCR (Odds Ratio and 95% CI; <i>p</i> value)			
	Univariable	<i>p</i>	Multivariable	<i>p</i>
Age	0.995 (0.965-1.025)	0.723		
Gender (male)	3.439 (1.644-7.192)	0.001	4.731 (1.986-11.271)	>0.001
Tumor length	1.357 (1.104-1.668)	0.004	1.402 (1.085-1.811)	0.010
cT (>2)	0.279 (0.088-0.887)	0.031	-	-
cN (+)	3.037 (0.890-10.360)	0.076	2.860 (0.741-11.045)	0.127
Interval nCRT-surg	1.015 (0.940-1.095)	0.712	-	-
Post-nCRT PLR	1.004 (1.001-1.007)	0.021	1.005 (1.001-1.009)	0.008

nCRT, neoadjuvant chemoradiotherapy; LARC, locally advanced rectal cancer; pCR, pathological complete response; CI, confidence interval; PLR, platelet-lymphocyte ratio.
Significant in univariable but not evidenced in the final model: post-nCRT NLR, post-nCRT SII.
Non-significant in univariable or multivariable analysis: tumor location, CRT regimen, blood parameters and scores not included in the table: hemoglobin, MCV, CEA, LMR, pre-nCRT NLR, PLR and SII.

Figure 1. ROC curve of value of post-nCRT PLR for prediction of pCR

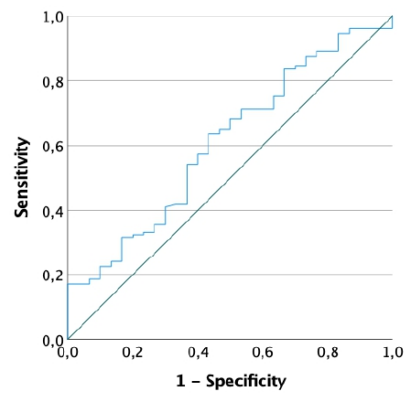
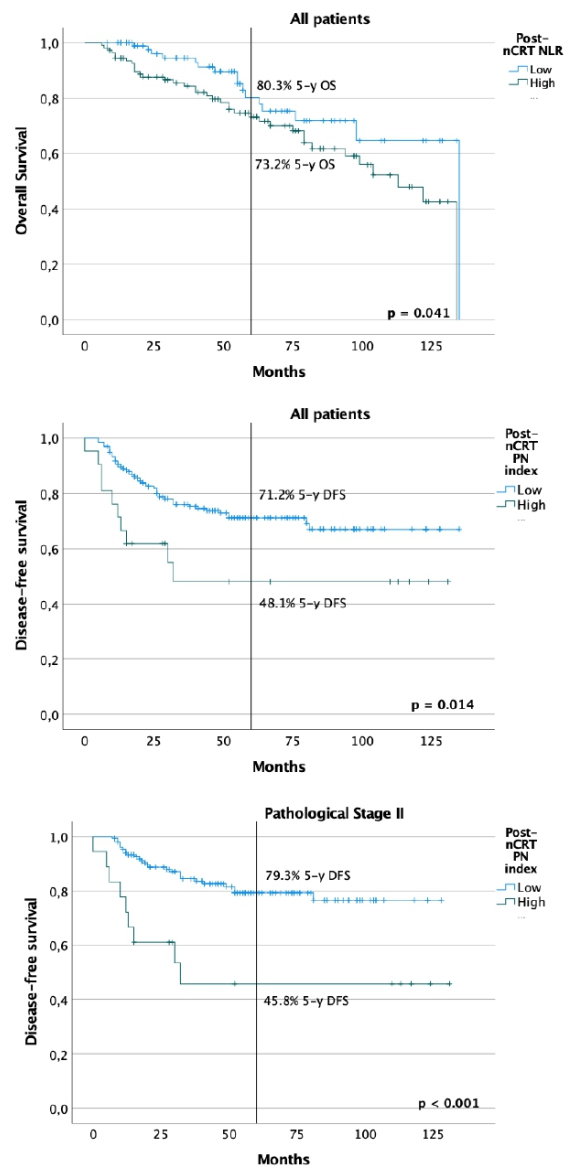


Table 3. Univariable and multivariable analysis (Cox Regression) of predictors of overall survival and disease-free survival

Variable	OS (HR and 95% CI; <i>p</i> value)				DFS (HR and 95% CI; <i>p</i> value)			
	Univariable	<i>p</i>	Multivariable	<i>p</i>	Univariable	<i>p</i>	Multivariable	<i>p</i>
Gender: Male	0.922 (0.575-1.479)	0.736			0.753 (0.467-1.216)	0.247		
Age	0.999 (0.979-1.020)	0.938			0.983 (0.964-1.002)	0.074		
Charlson	1.035 (0.905-1.184)	0.613	-	-	0.949 (0.824-1.093)	0.465		
BMI	0.933 (0.880-0.988)	0.018	-	-	0.980 (0.931-1.033)	0.454		
Clinical stage III	0.882 (0.521-1.491)	0.638	-	-	0.713 (0.423-1.200)	0.202	-	-
Dimensions	1.004 (0.898-1.123)	0.944	0.842 (0.716-0.991)	0.039	0.969 (0.865-1.086)	0.592	0.819 (0.718-0.935)	0.003
Interval treatment surgery (weeks)	1.022 (0.982-1.065)	0.283	-	-	0.991 (0.944-1.040)	0.711	-	-
Surgery APR	1.435 (0.906-2.271)	0.124	-	-	1.956 (1.246-3.072)	0.004	2.518 (1.406-4.511)	0.002
Surgical morbidity	1.277 (0.779-2.092)	0.333			1.252 (0.754-2.079)	0.384		
Pathological stage III	3.172 (2.007-5.012)	<0.001	4.763 (2.092-10.846)	<0.008	3.114 (1.980-4.897)	<0.001	2.584 (1.440-4.636)	0.001
Vascular invasion	2.708 (1.715-4.276)	<0.001	2.419 (1.162-5.034)	0.018	3.615 (2.272-5.753)	<0.001	-	-
Perineural invasion	3.270 (2.055-5.202)	<0.001	-	-	3.582 (2.253-5.692)	<0.001	2.400 (1.299-4.437)	0.005
R $\geq$ 1	4.066 (2.387-6.927)	<0.001	-	-	2.357 (1.334-4.165)	0.003	-	-
Non-pCR	2.201 (0.887-5.457)	0.089	-	-	4.559 (1.437-14.466)	0.010	-	-
CRM	0.698 (0.556-0.909)	0.008	-	-	0.878 (0.707-1.089)	0.237	1.216 (1.035-1.428)	0.017
DRM	1.006 (0.956-1.059)	0.806	-	-	1.033 (0.998-1.070)	0.068	-	-
No adjuvant CT	1.338 (0.836-3.267)	0.238	3.239 (1.530-6.855)	0.002	1.128 (0.700-1.816)	0.621	-	-
Pre-nCRT CEA	1.006 (1.002-1.010)	0.003	1.010 (1.004-1.016)	<0.001	1.008 (0.991-1.024)	0.367		
Pre-nCRT NLR	1.178 (1.019-1.361)	0.027	1.298 (1.032-1.632)	0.026				
Post-nCRT NLR	1.036 (1.003-1.070)	0.033	1.058 (1.008-1.111)	0.022				
Post-nCRT PN index					2.240 (1.289-3.894)	0.004	2.967 (1.470-5.991)	0.002

All remaining clinical data, such as nCRT regimen, blood parameters and scores studied in this work were not independent predictive factors of prognosis in this analysis. We did not include the parameter mesorectal complete excision, as about half of the patients had missing cases of these parameters. BMI, body mass index; APR, abdominal-perineal resection; pCR, pathological complete response; CRM; circumferential resection margin; DRM, distal resection margin; CT, chemotherapy; nCRT, neoadjuvant chemoradiotherapy; CEA, carcinoembryonic antigen; NLR, neutrophil-to-lymphocyte ratio; PN index, platelet-neutrophil index; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval.

Figure 2. Kaplan-Meier curves for overall survival and disease-free survival stratified by the scores retrieved from multivariable analysis (after R1-2 patients excluded)



Article 3

ctDNA on liquid biopsy for predicting response and prognosis in locally advanced rectal cancer: A systematic review.

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ctDNA on liquid biopsy for predicting response and prognosis in locally advanced rectal cancer: A systematic review

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ABSTRACT

Background: The management of locally advanced rectal cancer (LARC) requires a multidisciplinary approach, with an increasing interest for non-operative strategies. Liquid biopsy for obtaining circulating tumor DNA (ctDNA) can provide information on neoadjuvant chemoradiotherapy (nCRT) pathological response and cancer-specific prognosis, and therefore might be a promising guide for these treatments. **Methods:** A systematic review of the studies available in literature has been performed to assess the role of ctDNA as a predictive and prognostic biomarker in LARC patients.

Results: We retrieved 21 publications, of which 17 full-text articles and 4 abstracts. Results have been labelled into two groups: predictive and prognostic. Data about the usefulness of liquid biopsy in this setting is still inconclusive. However, baseline higher levels of longer fragments of cell-free DNA and integrity index, tumor-specific mutations and certain methylated genes could predict non-responders. Also, undetectable baseline ctDNA and decrease of common rectal cancer mutations throughout treatment (dynamic monitoring) were predictive factors of pathological complete response. The continuous detection of ctDNA in different timepoints of treatment (minimal residual disease) was consistently associated with worse prognosis.

Conclusions: ctDNA is a promising biomarker that could assist predicting treatment response to nCRT and prognosis in patients with LARC. The ideal methods and timings for the liquid biopsy still have to be defined.

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1. Background

Colorectal cancer is one of the most frequent cancers worldwide, with 1.88 million new cases and 0.92 new deaths reported in 2020 [1]. Rectal cancer accounts for about 25–30% of all colorectal cancers [2]. Locally advanced rectal cancer (LARC) patients are currently treated with a trimodality approach comprising neoadjuvant chemoradiation therapy (nCRT) and surgery with total mesorectal excision followed by adjuvant chemotherapy, when recommended. This combination of treatment is established by many randomized clinical trials as superior for several endpoints,

such as treatment compliance, acute and chronic toxicity, tumor downstaging, sphincter preservation, local recurrence rates, and patient survival [3,4].

Approximately 45% of patients respond to nCRT with tumor level downstaging [5], of which 24.4% achieve pathological complete response (pCR). The remaining patients exhibit incomplete or no response [6]. The identification of these patients, who do not respond to nCRT, could help clinicians to avoid unnecessary toxicities, decide for frontward surgery or, eventually, choose alternative treatment options. Besides, it is now widely accepted that pCR predicts a better prognosis and may confer a survival benefit to these patients [6]. With that in mind, some small prospective series published since the late 90's by Habr-Gama and colleagues [7,8] and several international series have proposed a non-operative management ("watch and wait"). These authors reported similar

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

oncologic outcomes in clinical complete response (cCR) patients followed by close active surveillance compared to those treated with radical surgery [9,10]. Likewise, in the International Watch & Wait Database, a large-scale registry of more than 1000 patients, there were reports of excellent survival and small risk of local unsalvageable disease recurrence [11]. Despite the extensive number of retrospective studies on this subject [12], the widespread use of the “watch and wait” approach is still limited by low sensitivity and specificity for prediction of response of the current imaging modalities, including positron emission tomography-computed tomography, magnetic resonance imaging and endoscopic ultrasound [13].

Concerning survival outcomes, rectal tumors radically operated carry a significant risk of distal recurrence mostly within the first 5 years, probably caused by micrometastatic spread. Stages II and III of Dukes have a risk of about 30% and 50%, respectively, [14]. The identification of the patients with high risk of recurrence could help decide on a change of regimen or dose of the adjuvant chemotherapy, or adopt alternative approaches, such as total neoadjuvant chemotherapy (TNT). Follow-up carried out by clinical examination, imaging and endoscopies aims to improve prognosis by early detection of local or distant recurrence. The analysis of serum protein levels, such as carcinoembryonic antigen (CEA), has proven to be insufficiently sensitive or specific [15,16].

In this respect, liquid biopsy for obtaining for circulating biomarkers might provide complementary information on rectal cancer chemoradiotherapy response and prognosis, on different moments throughout treatment [17,18]. Circulating biomarkers include circulating tumor cells (CTC), nucleic acids (cell-free DNA or cfDNA, microRNA), extracellular vesicles, proteins and metabolites, either originated from the cancer cells or from the host response and tissue microenvironment [19]. Unlike tissue-based biomarkers, blood-based molecular biomarkers allow non-invasive monitoring of response to therapy, through the assessment of the genetic heterogeneity of tumors and gathering of real-time measurements of biomarkers dynamic changes [19]. Mandel P. first described the presence of extracellular nucleic acids in the human bloodstream in 1948 [20]. It is currently known that cfDNA released from a tumor into the bloodstream has a typical half-life of 1.5–3h [21] and reflects several molecular alterations found in the tumor, such as mutations, methylation profile and microsatellite instability [19]. For this reason, this tumor specific cfDNA thence takes the name of circulating-tumor DNA (ctDNA) or methylated DNA (meth-DNA) [19,22]. The impact of cf/ctDNA in the diagnosis, prediction of response and prognosis of non-metastatic rectal cancer patients has been evaluated in a recent systematic review [23].

As the literature continues to grow and data remains controversial, a continuous update on the matter is of utmost importance. The purpose of this systematic review is to evaluate the current clinical potential of liquid biopsy of cfDNA/ctDNA, as a predictor of response to nCRT and prognosis, in LARC patients.

2. Methods

2.1. Publication search and study selection

A systematic literature review was performed according to PRISMA Statement Criteria [24]. The PubMed database was systematically reviewed as of February 7, 2021, using the Medical Subject Heading search terms: (“rectal” or “rectum” or “rectal cancer” or “LARC” or “rectal neoplasm”) and (“liquid biopsy” or “ctDNA” or “cfDNA” or “circulating tumor DNA” or “circulating free DNA” or “methylated DNA” or “DNA methylation”). Additional databases (Cochrane, Google Scholar, Wiley Online Library and Elsevier) were also searched for articles that examined the subject,

using the same search terms. Unpublished data presented as abstract in relevant international congresses, such as the American Society of Clinical Oncology (ASCO) and European Society of Medical Oncology (ESMO), were also systematically searched for. All retrieved studies were manually screened for relevant references.

Two authors (MM and DMP) independently examined the title, abstract and the full texts of potentially eligible studies; disagreements were resolved by discussion until a consensus was reached. We only included studies published in English in this systematic review. When multiple studies of the same patient population were identified, we included the published report with the largest sample size. Reviews, letters to the editor, and chapters of books were excluded. When data were unclear or incomplete, the corresponding author was contacted to clarify this data.

2.2. Inclusion and exclusion criteria

Studies included in this systematic review had to meet all the following criteria: (a) measurement of cfDNA/ctDNA in plasma/serum in patients with LARC submitted to long course nCRT, (b) description of therapeutic pathological response [pCR or tumor downstaging grade (TRG)], (c) description of survival data, (d) correlation of outcomes with cf/ctDNA and (e) inclusion of retrospective or prospective cohort studies.

All studies about stage I or metastatic rectal cancers were excluded, as were studies about other diseases (ulcerative colitis, endometriosis, benign polyps) and cancers other than rectal cancer. Also, when all stages of rectal cancer were analyzed simultaneously or when there were reports of clinicopathological end-points, these articles were excluded. When other parameter was analyzed other than liquid biopsy cf/ctDNA, such as tissue-based biomarkers, microRNA, CTCs, among others, we excluded these studies.

2.3. Data extraction and outcomes

The following data regarding each eligible study were recorded: the first author's surname, publication year, country of origin, total number of patients included, treatment, tumor regression grade (TRG), median follow-up, methods and assays used in the analysis. Pathologic response definition after nCRT varies in the different studies according to different TRG systems. However, in general, poor response correspond to residual tumor rate $\geq 75\%$, good response to residual tumor rate $< 50\%$ and complete response to residual tumor rate = 0%. All the data was organized in tables to allow a better comprehension of the findings and studies included in this review.

3. Results

3.1. Eligible studies

A total of 411 records were retrieved by a literature search of the PubMed, using different combinations of key terms. We also identified 125 additional records through other sources (Google Scholar, Elsevier Wiley Online Library, ESMO and ASCO). As displayed in the search flow diagram (Fig. 1), 21 studies were eligible and included in the systematic review: 17 full-text articles [22,25–40] and 4 abstracts presented at international congresses [41–44].

The sample sizes in all the eligible studies ranged from 4 to 194 patients. Overall, a total of 1499 patients were considered in this review. Ten of the studies were conducted in European populations (672 participants), whereas 6 were conducted in East Asian populations (584 participants), which corresponded to the majority of the population studied. All studies used long course nCRT before

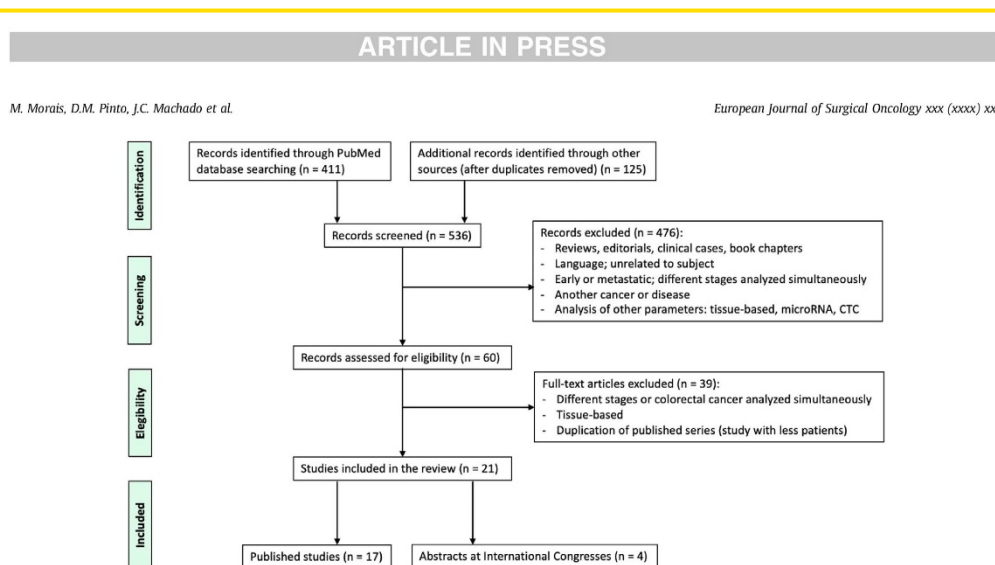


Fig. 1. Flow diagram of the systematic review process performed according to PRISMA Statement [24].

surgery, although 2 of the studies also started the treatment with induction chemotherapy [30,31]. One of the studies [22] used an additional $2 \times 5\text{Gy}$ brachytherapy tumor boost in its experimental arm and, in another study [36], some of the patients were proposed to short course radiotherapy, nCRT or induction chemotherapy. As for the methods of analysis, 11 studies employed polymerase chain reaction (PCR), 9 employed sequencing (either next-generation or whole-genome), one of them used fluorescence and another one genotyping.

3.2. Evidence synthesis

We intended to investigate the role of ctDNA/cfDNA on the prediction of treatment response and on the assessment of prognosis (in terms of disease recurrence or survival). A summary of the studies exploiting both outcomes are presented in Tables 1 and 2, respectively. In this review, a total of 18 studies exploited cfDNA/ctDNA as a predictor of treatment response and 15 studies exploited its role in survival and disease recurrence.

3.2.1. The use of cfDNA/ctDNA on the prediction of treatment response

Several groups analyzed the role of cfDNA/ctDNA on the prediction of treatment response after nCRT in LARC patients. In 2008, Zitt et al. showed for the first time an association between the concentration of cfDNA and pathological response in the 26 patients analyzed. Although these findings did not reach statistical significance, responders had lower levels of cfDNA, while non-responders had higher levels, both before and after nCRT [25]. By contrast, a Danish group quantified the total level of cfDNA, by fluorescence assay, in 123 LARC patients, and no differences were found in the baseline and post-treatment levels of cfDNA between pCR patients and poor responders [31]. Agostini and colleagues also assessed cfDNA on 67 LARC patients before and after nCRT. Like the previous study, baseline cfDNA levels were not associated with pathological response. However, the cfDNA integrity index, which corresponds to the ratio of longer cfDNA fragments over the levels of short fragments, was evaluated in this study. A difference between responders and non-responders was found in the cfDNA

integrity index collected after nCRT and in its change from before to after nCRT, which reveals that those longer fragments are more tumor-specific than short fragments. Also, the evaluation of the Alu 247 repeat, a biomarker of cfDNA integrity, showed a significant decrease over treatment in responders [26].

In line with the previous results, Sun et al. showed a significantly higher baseline longer fragments plasma cfDNA levels and a significant decrease of these fragments in plasma after nCRT, in 34 LARC patients. Moreover, they studied *MGMT* (O⁶-methylguanine-DNA methyltransferase) promoter methylation at baseline, which was higher in responders, although there was no reduction after treatment. This Chinese group also studied *KRAS* (Kirsten rat sarcoma viral oncogene homolog) mutation in the cfDNA, which was not predictive of response [27]. The same correlation between higher baseline methylated status of the promoter of *MGMT* and *ERCC1* (excision repair cross-complementing) genes with better response to nCRT was described by Shalaby et al. group [33], in a series of 42 patients, with an initial meth-DNA detection rate of 58% and 60%, respectively, for the aforementioned genes. Regarding dynamic changes before and after treatment, Chen et al. presented data on this aspect in the 2019 ASCO meeting. They detected ctDNA by analyzing the methylation status of *BCAT1* (Branched Chain Amino Acid Transaminase) or *IKZF1* (IKAROS Family Zinc Finger) gene through qPCR assay in 9 LARC patients. Five patients showed positivity for either or both of the methylated genes before nCRT and four of them showed a reduction after treatment, which was associated with non-statistical partial or complete responses [44].

Although works on meth-DNA seem promising, a study by Appelt et al., which included 146 patients, revealed no correlation between pre-treatment meth-DNA status (neuropeptide Y methylation) and pCR or TRG. We should, however, raise attention for the low detection of meth-DNA at baseline of only 20.5% (30 patients) with this particular epigenetic marker [22].

Carpinetti et al. were the first to demonstrate a decrease in ctDNA (detected by tumor-specific chromosomal rearrangements) in 4 patients that achieved response to nCRT, while cfDNA levels were negative both in patients with partial and complete pathological response [28]. Tie et al. analyzed multiple plasma samples of

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

Table 1

Studies exploring prediction of treatment response in locally advanced rectal cancer.

Reference	Year	N	Country	TRG	Assay	Main Findings
Zitt et al. [25]	2008	26	Austria	yp stage	qPCR	Responders and non-responders had comparable median plasma cfDNA values before (7.3 ng/ml vs. 3.6 ng/ml, $p = 0.693$) and after the end of chemoradiation (2.6 ng/ml vs. 1.0 ng/ml, $p = 0.340$). Baseline cfDNA levels were not associated with tumor response. Post-nCRT levels of cfDNA integrity index were significantly lower in responders ($p = 0.0009$). Median value of Alu 247 repeat and cfDNA integrity index (ratio of 400-/100-bp DNA fragments) decreased after nCRT in responders ($p = 0.005$ and $p = 0.005$, respectively).
Agostini et al. [26]	2011	67	Italy	Mandard [49]	qPCR	The good response group had significantly higher baseline 400-bp plasma cfDNA levels ($p < 0.05$) and ratio of 400-bp/100-bp DNA concentrations ($p < 0.05$). The 400-base pair (bp) DNA concentration and 400-/100-bp DNA ratio decreased significantly after chemoradiotherapy in the good responders ($p < 0.01$ and $p < 0.05$, respectively). Higher MGMT promoter methylation status at baseline DNA was associated with a better tumor response ($p = 0.04$), with no reduction after treatment, while the rate of KRAS mutation decreased in both groups after nCRT. Lack of correlation between normalization of ctDNA and amount of residual disease.
Sun et al. [27]	2014	34	China	Dworak [50]	qPCR	No difference between pre-treatment ctDNA positive and negative patients on pCR rate (15.4% vs. 10%, $p = 0.57$). No association between pre-nCRT or post-nCRT ctDNA status and pCR.
Carpinetti et al. [28]	2015	4	Brazil	Dworak [50]	WGS	Higher pre-treatment MGMT or ERCC1 methylation were correlated with better response to therapy.
Sclafani et al. [30]	2018	97	UK	RECIST [51]	ddPCR	No difference in baseline and after nCRT cfDNA levels between in pCR patients and poor responders.
Tie et al. [32]	2018	159	Australia	pCR [52]	NGS	Patients achieving pCR (25%) had the lowest values of pre-treatment ctDNA (median of 0.0), while patients with residual tumor had a median value of 0.11 ($p = 0.18$), with an AUCs of 0.67 (95% CI: 0.47–0.87). Similar results were observed for post-treatment ctDNA values.
Shalaby et al. [33]	2018	42	Egypt	Dworak [50]	qPCR	TP53 and APC mutations in pre-treatment samples is predictive of worse response ($p < 0.05$). A model based on support vector machine predicts groups with distinct TRG ($p = 0.03$). Four patients showed an immediate drop in detection of methylated BCAT1 and/or IKZF1 after one cycle of chemotherapy and remained at low concentration through the rest of the cycles. These patients have shown partial or complete response to the treatment regimen.
Schou et al. [31]	2018	123	Denmark	Mandard [49]	Direct fluorescence	No correlation between meth-ctDNA status at baseline and complete ($p = 0.76$) or major ($p = 1.0$) tumor regression.
Ravenda et al. ^a [42]	2018	28	Italy	pCR [52]	NGS	In the 3 (13%) cases of pCR, ctDNA was detectable pretreatment and became undetectable from mid nCRT onwards.
Yang et al. ^a [43]	2019	119	China	pCR [52]	NGS	In all patients, ctDNA was strongly reduced or eliminated from plasma by the end of the first week of nCRT, with no correlation with TRG.
Chen et al. ^a [44]	2019	9	USA	NA	qPCR	Decrease of ctDNA levels from baseline to after preoperative treatment was an independent predictor of complete response to preoperative therapy ($p = 0.0276$). The predictive classifier based on promoter profiling of pre-treatment ctDNA had an AUC of 0.89 (95% CI 0.83–0.94), to discriminate individuals with pCR from non-pCR patients.
Appelt et al. [22]	2020	146	Denmark	Mandard [49]	ddPCR	None of the 29 patients with ypCR had preoperative ctDNA detected. Preoperative ctDNA positive rate was significantly lower in the well responded patients ($p < 0.001$) and lower in ypCR than non-ypCR group ($p = 0.02$). POLD1 mutation and higher tumor mutation burden may potentially be used as the predictor of good tumor response.
Khakoo et al. [34]	2020	47	UK	Mandard [49]	ddPCR	Patients with undetectable preoperative ctDNA presented significantly higher overall margin-negative, node-negative resection rate ($p = 0.028$) and more favorable neoadjuvant rectal scores (surrogate endpoint to pCR) ($p = 0.029$).
Pazdirek et al. [35]	2020	36	Czech Republic	Dworak [50]	Singleplex PCR	
Murahashi et al. [36]	2020	85	Japan	Dworak [50]	Amplicon-based deep sequencing	
Guo et al. [37]	2020	194	China	RECIST [51]	WGS	
Zhou et al. [38]	2021	106	China	CAP grading system [53]	NGS	
McDuff et al. [40]	2021	29	USA	Becker [54]	NGS and ddPCR	

^a Published as abstract; TRG: tumor regression grade; ctDNA: cell-free DNA; nCRT: neoadjuvant chemoradiotherapy; ctDNA: circulating-tumor DNA; pCR: pathological complete response; RECIST: Response evaluation criteria in solid tumors; NA: not available; CAP: College of American Pathologists.

159 LARC patients through next-generation sequencing (NGS), searching for tumor-specific mutations or the mutations with the highest variant allele frequency (VAF). ctDNA was detected in 77% of plasma samples before treatment, 8% during nCRT and 12% postoperatively. No significant association was found between reduction of ctDNA after nCRT and pCR [32]. In another study by Pazdirek et al. group, in which seven patients (7/33, 21.2%) presented positive ctDNA prior to therapy, also no correlation was found between dynamic changes of ctDNA and TRG [35]. Nevertheless, in a more recent Japanese study of 85 patients, using amplicon-based deep sequencing, a fall of ctDNA levels from before to after treatment, was a predictor of pCR. Murahashi et al. detected ctDNA in 57.6% and 22.3% of samples at baseline and after preoperative treatment, respectively, which was also significantly different ($P = 0.0003$) [36].

As for timepoint analysis of ctDNA, initially no difference was found between baseline ctDNA positive and negative patients and rate of pCR on a British study of 97 patients. They used digital droplet PCR (ddPCR) to detect variants in genes of interest, such as

KRAS or BRAF (v-raf murine sarcoma viral oncogene homolog B1) mutations, with a 74% detection at baseline [30]. Likewise, another study of 28 patients, presented at the 2018 ESMO meeting, showed similar results, although there was a trend for lower pre- and post-treatment ctDNA in pCR patients. These patients' plasma was analyzed by NGS for detection of KRAS, BRAF and PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) mutations [42]. In a study of 47 patients, Khakoo et al. showed a trend for dramatic reduction of ctDNA levels, from baseline onward in 3 patients with pCR, although these results were not statistically significant [34].

However, more recent and larger works are gathering positive associations with these outcomes. Yang et al. reported a worse prognosis when TP53 (tumor protein 53) and APC (adenomatous polyposis coli) gene mutations, analyzed through NGS gene panels, were present in pre-treatment samples of 119 patients. Detectability of baseline mutations during nCRT also significantly decreased from TRG3 to TRG0 group [43]. A Chinese study of Guo et al., on 194 patients, also achieved an excellent discrimination of

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

Table 2
Studies exploring prediction of survival and disease recurrence in locally advanced rectal cancer.

Reference	Year	N	Country	Follow-up (median in months)	Assay	Main Findings
Carpinetti et al. [28]	2015	4	Brazil	Between 21 and 80	WGS	ctDNA changes after surgery predict tumor progression.
Boysen et al. [29]	2017	75	Sweden	39	ddPCR	The pre-operative median level for patients with recurrent disease was 13,000 copies/mL compared to 5200 copies/mL for non-recurrent patients ($p = 0.08$).
Sclafani et al. [30]	2018	97	UK	60	ddPCR	Pre-treatment ctDNA positive and ctDNA negative patients showed no difference in terms of progression-free and overall survival.
Tie et al. [32]	2018	159	Australia	24	NGS	Significantly worse DFS was seen if ctDNA was detectable after chemoradiotherapy (HR 6.6; $p < 0.001$) or after surgery (HR 13.0; $p < 0.001$). Postoperative ctDNA detection was predictive of recurrence irrespective of adjuvant chemotherapy use (chemotherapy: HR 10.0; $p < 0.001$; without chemotherapy: HR 22.0; $p < 0.001$).
Schou et al. [31]	2018	123	Denmark	55	Direct fluorescence	Baseline ctDNA levels above the 75th quartile had a higher risk of recurrence and shorter time to recurrence (HR = 2.48, $p = 0.007$). During follow-up, the association remained significant regardless of time point for sample analysis.
Toomey et al. ^a [41]	2018	27	Ireland	48	Genotyping	There was no recurrence in any patient with negative ctDNA post-surgery.
Yang et al. ^a [43]	2019	119	China	24	NGS	Detection of pre-treatment mutations after completion of nCRT was significantly associated with worse DFS ($P < 0.05$). The presence of TP53 mutations is predictive of treatment outcome and shows a statistically significant worse DFS (2 years) (54.3% VS 87.0%, $P = 0.0005$).
Chen et al. ^a [44]	2019	9	USA	NA	qPCR	One patient with continuous high levels of methylated <i>BCAT1</i> and <i>IKZF1</i> post-treatment have recurred two months after surgery.
Appelt et al. [22]	2020	146	Denmark	127	ddPCR	Patients with baseline meth-ctDNA had significantly worse 5-year OS (47% vs. 69%; HR = 2.08; 95% CI, 1.23–1.51). Increased quantitative load was highly significant for worse outcomes. Total ctDNA showed no association with survival outcomes.
Khakoo et al. [34]	2020	47	UK	26.4	ddPCR	No difference in MFS by patients with detectable or undetectable ctDNA pretreatment or mid nCRT. MFS was shorter in patients with detectable ctDNA after completing nCRT (HR 7.1; $p < 0.001$), persistently detectable ctDNA pre and mid nCRT (HR 3.8; $p = 0.02$), and pre, mid, and after nCRT (HR 11.5; $p < 0.001$). In patients with detectable ctDNA, a fractional abundance threshold of $\geq 0.07\%$ mid nCRT or $\geq 0.13\%$ after completing nCRT predicted for metastases. All 3 patients with detectable ctDNA post-surgery relapsed compared with none of the 20 patients with undetectable ctDNA ($p = 0.001$).
Pazdirek et al. [35]	2020	36	Czech Republic	at least 36	Singleplex PCR	The ctDNA positivity (21.2%) before treatment reduced post-operative DFS and OS ($p = 0.015$ and $p = 0.010$, respectively).
Murahashi et al. [36]	2020	85	Japan	80	Amplicon-based deep sequencing	Postoperative ctDNA ($p = 0.0127$) and CEA (0.0105) were independent prognostic markers for risk of recurrence after surgery, with a combined analysis having cumulative effects on DFS ($P = 1.0 \times 10^{-16}$).
Zhou et al. [38]	2021	106	China	18.8	NGS	ctDNA positivity at all four time points (baseline, during nCRT, pre-surgery and post-surgery) was associated with a shorter MFS ($p < 0.05$). Median VAF of mutations in baseline ctDNA was a strong independent predictor of MFS (HR = 1.27, $P < 0.001$).
Ji et al. [39]	2021	46	China	40	NGS	There was no significant correlation between ctDNA levels and tumor recurrence or overall survival at baseline, post nCRT or post operation. Low bTMB level (≤ 10 Mb) at baseline was positively correlated with DFS ($p = 0.036$). Following nCRT, the patients with decreasing TMB tend to have a longer median RFS ($p = 0.022$). High bTMB level (> 10 Mb) after surgery was negatively correlated with DFS ($p = 0.026$).
McDuff et al. [40]	2021	29	USA	20	NGS	Patients with detectable postoperative ctDNA experienced poorer DFS (HR 11.56; $p = 0.007$).

^a Published as abstract; ctDNA: circulating-tumor DNA; cfDNA: cell-free DNA; nCRT: neoadjuvant chemoradiotherapy; NA: not available; DFS: disease-free survival; OS: overall survival; MFS: metastasis-free survival; bTMB: blood-based tumor mutational burden.

pCR patients through global (fragment profiles at 5-Mb windows, sub-compartments and mitochondrial DNA copy number) and local chromatin variables (promoter profiling of several genes) at baseline [37]. Furthermore, Zhou et al., another Chinese study of 106 patients, reported that ctDNA levels at baseline could discriminate patients with good pathological response and pCR, and also that POLD1 mutation and higher tumor mutation burden could also help predict good response [38]. In this study, ctDNA was detected in 75%, 15.6%, 10.5%, and 6.7% of cases at the four time points, respectively. Finally, McDuff et al. reported a higher rate of R0-node negative resections and more favorable neoadjuvant rectal scores (a surrogate endpoint of pCR) among 20 patients with undetectable preoperative ctDNA. This group had a non-significant higher pCR

rate (24% vs. 11%) [40].

3.2.2. cfDNA/ctDNA as a prognostic tool for disease recurrence or survival

Boysen and colleagues were the first to observe that pre-operative cfDNA levels could help predict recurrence in 75 LARC patients, with a follow-up of 39 months [29]. Schou et al. also showed a significant association, this time, between higher baseline cfDNA level (measured through a fluorescence assay) with disease-free survival (DFS) in 123 LARC patients. This association remained significant throughout the different timepoints and a trend for worse overall survival (OS) was also witnessed [31]. On the other hand, a recent study from 2021, by Ji et al. could not find

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

any association between cfDNA levels at different timepoints and survival outcomes, although this study had an inferior number of patients and follow-up (46 patients followed through 40 months) [39]. Also, Appelt et al. failed to show any association between baseline cfDNA levels and survival outcomes, in a 146 patients' sample, followed for 127 months [22].

Carpinetti et al. began measuring ctDNA in 4 LARC patients to monitor disease response and recurrence. Two patients with persistent positive level of ctDNA, during follow-up, developed liver metastasis and other 2 patients that showed a drop in ctDNA levels after CRT, had no recurrence and no more evidence of ctDNA in plasma [28]. Although these were promising results, a study conducted on 97 LARC patients by Sclafani et al. did not find a significant association between ctDNA levels and progression free survival (PFS) or OS [30]. Also, an Irish study of 27 patients Toomey, found only a trend towards less recurrence in patients with negative ctDNA post-surgery, measured by genotyping [41].

With these results in mind, a series of studies have continued to analyze liquid biopsies and have proven significance to ctDNA in disease-free and overall survival. Tie and colleagues observed an increased risk of recurrence in patients with ctDNA detectable after nCRT or surgery, irrespective of pathological risk level or of adjuvant therapy, with an estimate 3 years DFS of 33% vs 87%, in 150 patients followed for a median of 24 months [32]. These results are shared by Murahashi et al. and McDuff et al., in which studies, post-surgery ctDNA was a strong prognostic factor of worse recurrence [36,40]. Furthermore, Yang et al. revealed, at the 2019 ASCO meeting, a significant association in 119 patients between persistence of pre-treatment mutations in ctDNA after completion of nCRT and worse DFS [43]. As for other timepoints, Pazdirek et al., revealed that also baseline ctDNA positivity was associated with worse outcomes (DFS and OS) [35]. Khakoo et al. also showed that persistence of ctDNA at mid CRT or detection of ctDNA at the end of CRT were associated with worse metastasis free survival (MFS), with detection rates of 74% pretreatment, 21% mid-CRT, 21% after completing CRT, and 13% after surgery [34]. These results have been replicated by Zhou et al., in their study of 106 patients, that revealed that ctDNA positivity at all 4 timepoints (baseline, during and after nCRT, and post-surgery) was associated with shorter MFS [38]. Ji et al. have also shown that blood tumor mutational burden (bTMB), through analysis of mutational signature of a Catalogue of Somatic Mutations in Cancer (COSMIC) [45], at different time points (baseline, after nCRT and after surgery) was strongly associated with RFS [39].

As far concerns meth-DNA, in Chen et al. work presented at 2019 ASCO meeting, one patient that showed persistent high levels of methylated genes after nCRT, recurred in two months after surgery [44]. Appelt et al. explored this result further and came to the conclusion, in their 146 patients sample followed through a median of 127 months, that baseline meth-DNA positivity was strongly associated with worse 5-year OS, and was significantly worse with increased quantitative load [22].

4. Discussion

This review explores several studies that support the use of liquid biopsy through analysis of cf/ctDNA in LARC, that could complement the assessment of treatment response and survival. Contrasting data have been found on the reliability of cf/ctDNA as a clinical tool. In order for liquid biopsies to become suitable as a tool for predicting treatment response and evaluating prognosis, to change current strategies and improve clinical practice, further well-conducted experimental studies are needed. With the intent to guide these future studies, the authors of this review provide a comprehensive synopsis of the results published in the literature,

in Tables 3 and 4.

4.1. Tumor regression grade

Ten studies described an association of cf/ctDNA with tumor regression grade. The studies that described an association with TRG had a total sample that ranged from 42 to 119 patients and a baseline detection rate of ctDNA above 60% [26,27,33,38,43]. The dynamic changes throughout therapy, of increasing longer fragments of cfDNA, median value of Alu 237 repeat and integrity index were linked to non-responders [26,27]. Baseline evaluation of mutations from tissue sample in liquid biopsies, blood based tumor mutational burden (bTMB) [38], *TP53* or *APC* mutations [43] (negative association) and *MGMT* or *ERCC1* genes methylations [27,33] (positive association) could also predict TRG in the studies evaluated. These studies used predominantly qPCR and NGS as assays in their analyses. As ctDNA corresponds only to a very small proportion of cfDNA, very sensitive and reliable detection methods are required. Real-time PCR (qPCR) is a targeted approach that searched for a limited number of pre-determined genes at a time, has a low cost and is easy to perform, but has a low sensitivity. DdPCR carries the same features as qPCR, but with a higher sensitivity and specificity. An untargeted approach can also be employed with sequencing methods. These methods can identify structural rearrangements, chromosomal copy-number changes, and structural alternations of ctDNA in circulation. They allow more genes to be analyzed at a time (with amplicon strategies being even more sensitive), but at a higher cost [46].

Although repeatedly tested, baseline cfDNA total levels [25,26] and dynamic changes in *KRAS* mutations [27,35] did not have an association with TRG. Also, baseline NPY hypermethylation, tested in 146 patients, but with only 20.5% detection at baseline, showed no association with this outcome [22].

4.2. Pathological complete response

As for pathological complete response, a total of 11 studies provided information on this outcome. Studies that described an association with this outcome ranged between 29 and 194 patients, with a baseline ctDNA detection rate above 57.6% [36–38,40]. Most studies analyzed ctDNA through NGS or WGS. Baseline specific mutations from tissue samples [38,40] and pre-treatment promoter profiling of cfDNA [37] permitted prediction of pCR. Furthermore, dynamic changes (until just before surgery) of several common mutations, such as *TP53*, *KRAS*, *APC*, *PIK3CA*, etc. [36], could predict complete response, in opposition to the outcome previously mentioned, TRG. These groups observed a correlation between undetectable baseline ctDNA and pathological downstage, which raises the conviction of using ctDNA as a tool to guide patient selection for a "watch and wait" strategy.

Similar to tumor regression grade outcome, a study on baseline cfDNA general levels (through direct fluorescence) [31], was inconclusive. Possibly, levels cfDNA are not sensitive enough to rule out the presence of minimal residual disease. Also, multiple studies on pre- and post-treatment measurement of *KRAS/BRAF* mutations [30,42] and baseline NPY hypermethylation [22], showed no association with pathological complete response. Sclafani et al. explains that undetectable plasma *KRAS/BRAF* mutations could be due to either the absence of micrometastatic disease, the necessity of more sensitive assays or that maybe tumors harboring these mutations are less likely to release DNA in blood [30].

4.3. Survival outcomes

Fifteen studies approached the association of cf/ctDNA and

Table 3
Summary

^a Published as abstract; cfDNA: cell-free DNA; nCRT: neoadjuvant chemoradiotherapy; MAF: mutation allele frequency.

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

Table 4
Summary of findings for prognosis.

Outcomes	Method	n	Follow-up (median, months)	Assay	Timing
	Description				
Association with overall survival	ctDNA (mutations): ◦ Pazdirek et al. [35]: KRAS, BRAF, PIK3CA, CRNNB1, APC, TP53 meth-DNA(methylations): ◦ Appelt et al. [22]: NPY, quantitative load	36 146	36 127	ddPCR: ◦ Appelt et al. [22] Singleplex PCR: ◦ Pazdirek et al. [35]	Pre-nCRT: ◦ Appelt et al. [22]: 6 days before ◦ Pazdirek et al. [35]
Association with disease-free survival	cfDNA: ◦ Schou et al. [31]: levels: 75th quartile ctDNA: ◦ Tie et al. [32]: one mutation from tissue or one with higher MAF ◦ Yang et al. ^a [43]: TP53, APC ◦ Khakoo et al. [34]: 3 somatic variants, fractional abundance; endpoint MFS ◦ Pazdirek et al. [35]: KRAS, BRAF, PIK3CA, CRNNB1, APC, TP53 ◦ Murahashi et al. [36]: TP53, KRAS, APC, PIK3CA, FBXW7, NRAS, GNAS, SMAD4, MAP2K1, ERBB2, BRAF, AKT1, CTNNB1 and EGFR ◦ Zhou et al. [38]: mutation from tissue sample, VAF; endpoint MFS ◦ Ji et al. [39]: bTMB levels ◦ McDuff et al.: specific somatic mutations	47 159 119 26,4 36 85 106 46 29	55 24 24 26,4 36 80 18,8 40 20	PCR (dd or Singleplex): ◦ Khakoo et al. [34], Pazdirek et al. [35] Direct fluorescence: ◦ Schou et al. [31] NGS: ◦ Tie et al. [32], Yang et al. ^a [43], Zhou et al. [38], Ji et al. [39], McDuff et al. [40] Amplicon-based deep sequencing: ◦ Murahashi et al. [36]	Pre-nCRT: ◦ Schou et al. [31], Pazdirek et al. [35], Zhou et al. [38], Ji et al. [39] Post-nCRT: ◦ Tie et al. [32]: 4–6 weeks after ◦ Yang et al. ^a [43] ◦ Khakoo et al. [34]: 4–12 weeks after Dynamic changes: ◦ Schou et al. [31]: one year after surgery and last available time point ◦ Khakoo et al. [34]: persistence pre-nCRT 4 weeks before, mid-nCRT 3–4 weeks from start and after nCRT 4–12 weeks ◦ Zhou et al. [38]: pre-nCRT, mid-nCRT one cycle after, post-nCRT 7 weeks after, post-surgery 1 month after ◦ Ji et al. [39]: after nCRT Post-surgery: ◦ Tie et al. [32]: 4–10 weeks after ◦ Khakoo et al. [34]: 4–12 weeks after ◦ Murahashi et al. [36]: 12 weeks after ◦ Ji et al. [39]: 1 day after ◦ McDuff et al. [40]
No association with overall survival or disease-free survival	cfDNA: ◦ Appelt et al. [22] ◦ Boysen et al. [29]: endpoint DFS ctDNA: ◦ Sclafani et al. [30]: KRAS, BRAF ◦ Ji et al. [39]: levels ◦ Khakoo et al. [34]: 3 somatic variants, fractional abundance; endpoint MFS ◦ Carpinetti et al. [28]: endpoint DFS ◦ Toomey et al. [41]: KRAS, MAF; endpoint DFS meth-DNA: ◦ Chen et al. ^a [44]: BCAT1, IKZF1; endpoint DFS	146 75 97 46 47 4 27 9	127 39 60 40 47 21–80 48 –	dd/qPCR: ◦ Sclafani et al. [30], Appelt et al. [22], Khakoo et al. [34], Boysen et al. [29], Chen et al. ^a [44] NGS or WGS: ◦ Ji et al. [39], Carpinetti et al. [28] Genotyping: ◦ Toomey et al. [41]	Pre-nCRT: ◦ Sclafani et al. [30], Appelt et al. [22], Ji et al. [39], Boysen et al. [29] ◦ Khakoo et al. [34]: pre- and mid-nCRT 3–4 weeks from start Post-nCRT: ◦ Ji et al. [39], Toomey et al. [41], Chen et al. ^a [44] Dynamic changes: ◦ Carpinetti et al. [28]: several measurements after nCRT Post-surgery: ◦ Ji et al. [39]

^a Published as abstract; ctDNA: circulating-tumor DNA; cfDNA: cell-free DNA; nCRT: neoadjuvant chemoradiotherapy; MAF: mutation allele frequency; VAF: variant allele frequency; DFS: disease-free survival; MFS: metastasis-free survival.

survival outcomes (RFS, MFS and OS). The number of patients in studies with an association with outcomes ranged between 29 and 159 patients and the median follow-up ranged between 18.8 and 127 months [22,31,32,34–36,38–40,43]. Studies analyzing tumor tissue-specific mutations [34,36,38,40], common mutations mentioned above [35,36,43] and tumor mutational burden [39], through NGS, and showing persistence of ctDNA in 4 time-points (baseline, dynamic changes throughout CR, post-CRT and post-surgery), could predict survival outcomes. Tie et al. explains that ctDNA detected at baseline is being released by the primary tumor and consequentially is diagnostic and not prognostic. Only when the tumor is removed, the persistence of ctDNA reflects residual micrometastatic disease and a potential recurrence risk [32]. Nevertheless, some studies on KRAS/BRAF mutations or in certain specific timepoints [30,34,39,41], showed no association. Similar outcomes have been reached before in the setting of resected colon cancer, where persistence of ctDNA after surgery or adjuvant chemotherapy was correlated with shorter RFS [47,48]. Baseline cfDNA levels above 75th quartile [31], could also predict outcomes,

although repeated studies on cfDNA total levels did not [22,29]. Unlike pathological response, baseline NPY hypermethylation could predict overall survival [22].

The limitations of this review are mostly related to the possibility of publication bias, relatively small sample size of the studies, heterogeneity of techniques used for liquid biopsy, many different timings of plasma samples, differences in the treatment context (e.g. induction chemotherapy, brachytherapy), the use of many tumor pathological grading systems and variability in the duration of follow-up. Despite these limitations, this systematic review was able to study a large number of cases that were pooled from the different studies and a comprehensive synopsis of all the published literature on the subject was performed, which represents significant strengths of this review.

Although plenty of data in the work still needs clarification and validation, this systematic review supports ctDNA as a useful predictive factor for assessing treatment response to nCRT and evaluate prognosis in patients with LARC. The ideal method and timing for the liquid biopsy is still a matter of debate and should be

clarified in future studies, with larger sample sizes, more precise methods and more comprehensive study designs.

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Author contributions

Conception and design: MM. Analysis of data: MM and DMP. Manuscript preparation: MM. Manuscript review: MM, DMP, JCM and SC.

Registration

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Appendix A. Supplementary data

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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M. Morais, D.M. Pinto, J.C. Machado et al.

European Journal of Surgical Oncology xxx (xxxx) xxx

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Article 4

Evaluation of ctDNA in the prediction of response to neoadjuvant therapy and prognosis in LARC patients: a prospective study

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Article

Evaluation of ctDNA in the Prediction of Response to Neoadjuvant Therapy and Prognosis in Locally Advanced Rectal Cancer Patients: A Prospective Study

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Abstract: “Watch and wait” is becoming a common treatment option for patients with locally advanced rectal cancer (LARC) submitted to neoadjuvant treatment. However, currently, no clinical modality has an acceptable accuracy for predicting pathological complete response (pCR). The aim of this study was to assess the clinical utility of circulating tumor DNA (ctDNA) in predicting the response and prognosis in these patients. We prospectively enrolled a cohort of three Iberian centers between January 2020 and December 2021 and performed an analysis on the association of ctDNA with the main response outcomes and disease-free survival (DFS). The rate of pCR in the total sample was 15.3%. A total of 24 plasma samples from 18 patients were analyzed by next-generation sequencing. At baseline, mutations were detected in 38.9%, with the most common being *TP53* and *KRAS*. Combination of either positive magnetic resonance imaging (MRI) extramural venous invasion (mrEMVI) and ctDNA increased the risk of poor response ($p = 0.021$). Also, patients with two mutations vs. those with fewer than two mutations had a worse DFS ($p = 0.005$). Although these results should be read carefully due to sample size, this study suggests that baseline ctDNA combined with mrEMVI could potentially help to predict the response and baseline ctDNA number of mutations might allow the discrimination of groups with different DFS. Further studies are needed to clarify the role of ctDNA as an independent tool in the selection and management of LARC patients.

Keywords: ctDNA; next-generation sequencing; locally advanced rectal cancer; neoadjuvant chemoradiation; prediction of response; disease-free survival

1. Introduction

Colorectal cancer is a major health burden worldwide, with 1.88 million new cases and 0.92 million new deaths reported in 2020 [1]. Rectal cancer represents one third of the cancers of the large intestine. Preoperative chemoradiotherapy (nCRT) followed by radical surgery is the standard of care for patients with locally advanced rectal cancer (LARC) due to improvement in local recurrence rates [2], downstaging of the tumor [3], better sphincter preservation [4] and a safer closer distal margin at resection [5,6]. However, novel approaches are emerging, such as total neoadjuvant therapy (TNT) followed by surgery, in order to improve these outcomes [7], treating occult micrometastases several months earlier, increasing treatment compliance, enhancing the efficacy of chemotherapy and ultimately improving survival [8].

A pathological complete response (pCR) to neoadjuvant treatment, in which no viable tumor cells are present in the surgical specimen, is achieved in 15–20% of patients [9]. These

patients benefit from improved local and distant disease control and could potentially avoid the complications of surgery [10]. Surgery has been associated with long-term sequelae, including urinary and sexual dysfunctions, fecal incontinence, temporary or permanent stomas and immediate post-operative risks, such as bleeding, infection and anastomotic leak [11]. The elderly population, in which there is a higher prevalence of mismatch repair-deficient cancers with microsatellite instability, larger and locally invasive disease and lower lymph node metastasis, is even more prone to severe post-operative complications, especially those with pT4 disease [12]. Novel approaches could benefit these patients. The “watch and wait” approach was popularized by Habr-Gama et al. [10] in 2004 for patients who achieve complete clinical response (cCCR). These patients do not receive any surgery and are maintained under close surveillance. However, to this date, none of the clinical tools available, including endorectal ultrasound or magnetic resonance imaging (MRI), has an acceptable accuracy for predicting pCR [13].

A promising biomarker of pCR is the circulating tumor DNA (ctDNA) collected in liquid biopsies, which is secreted from cancer cells into the peripheral blood as a result of cell apoptosis, necrosis or via exosomes [14,15] and represents a small fraction (<1% in some studies) of the circulating free DNA [16]. This biomarker has shown application in monitoring treatment response, cancer recurrence and drug resistance in colorectal cancer patients [17–19]. Due to its short turnover time, ctDNA may provide a real-time information about the disease [20]. Some limitations have been attributed to ctDNA, such as the generally low concentrations extracted from plasma, background noise from non-tumor cells or induced by inflammation and trauma [21] and exacerbation of these by improper pre-analytical handling [22]. Nevertheless, it was reported that ctDNA was a more sensitive method than CEA or radiological imaging in detecting disease progression [17,23,24] and that ctDNA might circumvent the limitations of tissue biopsies, such as invasiveness and not representing the whole heterogeneity of the disease [25]. The next-generation sequencing (NGS) technique uses commercially available kits and is easy to implement in a laboratory. It has the advantage of allowing the characterization of different genes in a single run using a smaller quantity of DNA for obtaining a large amount of molecular data and for its good sensitivity (0.1%) in comparison with some of the most common polymerase chain reaction (PCR)-based methodologies.

As examined before in a systematic review [26], results are still very inconsistent across studies about which mutations, methylations, methods or timings are more accurate for predicting response and prognosis. The aim of this study was to add some information to the literature on the clinical utility of baseline and serial ctDNA analysis in predicting response to neoadjuvant treatment and prognosis in LARC patients.

2. Results

2.1. Study Enrollment

We prospectively enrolled 30 patients with LARC who met inclusion criteria, after excluding patients who did not accept to participate in the study ($n = 6$), in which disease progressed ($n = 9$) or with missing baseline samples ($n = 7$). The rate of pCR in the total sample was 15.3% (8/52 patients). Figure 1 shows the flowchart of patient selection for NGS analysis. After obtaining response outcomes, the patients were divided into groups of response to therapy for further analysis. Eight patients achieved pCR, two patients achieved cCR, four patients had a good response and sixteen patients presented a poor response. From these, only seven patients had two samples collected before and after neoadjuvant treatment. All patients with a complete or good response were selected for NGS analysis: however, only four patients with baseline and post-treatment samples from the poor response group were chosen for the analysis as controls for the main outcomes of the study (complete and good response) due to budget restrictions. We ended up with a cohort of 18 patients.

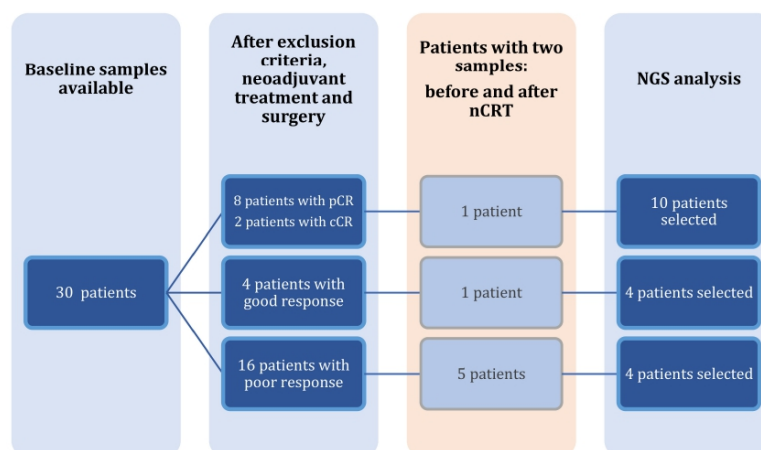


Figure 1. Study flowchart of selection of patients for NGS analysis.

2.2. Clinicopathological Features

The median age of patients was 65 years old (38–86). Eight patients (44.4%) were males and ten (55.6%) were females. The median distance from the anal verge was 6 cm (2–12). Pretreatment CEA median levels were 2.6 ng/mL and pretreatment mrEMVI was positive in nine (50%) of patients. Fourteen patients (77.8%) were diagnosed as clinical stage III and three patients (16.7%) as clinical stage II; two patients (11.1%) had clinical T4 and sixteen (88.9%) clinical T3. Twelve patients (66.7%) received standard nCRT and six received short-course radiotherapy and neoadjuvant chemotherapy. After preoperative therapy, two patients achieved cCR (ycT0N0M0), nine patients had a good response and five presented with a poor response in reevaluation MRI. Twelve patients (66.7%) were submitted to anterior rectal resection, whereas four (22.2%) had an abdomino-perineal resection performed. The median interval from completing preoperative therapy to surgery was 10 weeks (8–26). After surgery, eight patients had pCR and four patients achieved good response (TRG 1). One patient had ypT1 stage, one had ypT2 stage, six presented with ypT3 stage and three patients had positive ypN post-operatively. Furthermore, two patients (12.5%) presented mucinous histology, three (16.7%) had venous invasion and one (5.6%) had perineural invasion. Five patients (27.8%) were treated with adjuvant chemotherapy and at a median follow-up of 14 months, we observed three (16.7%) systemic recurrences. Table 1 shows the clinico-pathological features of this sample.

Table 1. Clinico-pathological features of the patients.

	N	%
Age, years, median	65 (38–86)	
Sex		
Male	8	44.4
Female	10	55.6
BMI, kg/m ² , median	25.6 (16.9–30.7)	
Smokers	6	33.3%
Tumor location from the anal verge, median cm	6 (2–12)	
Pretreatment CEA, median ng/mL	2.6 (1–21)	
Pretreatment positive ctDNA	7	38.9

Table 1. Cont.

	N	%
Post-treatment positive ctDNA	4	66.7
Clinical T		
cT3	16	88.9
cT4	2	11.1
Clinical stage		
II	3	16.7
III	14	77.8
mrEMVI positive	9	50.0
Neoadjuvant treatment		
nCRT	12	66.7
SCRT+TNT	6	33.3
Interval from therapy to surgery, median weeks	10 (8–26)	
MRI response		
Complete (mrTRG1)	2	11.1
Good (mrTRG2)	9	50.0
Poor (mrTRG3–5)	5	27.8
Surgery		
Anterior resection	12	66.7
APR	4	22.2
Pathological T		
ypT0–2	10	55.6
ypT3–4	6	33.3
Pathological N		
ypN0	13	72.2
ypN1–2	3	16.7
Mucinous histology	2	12.5
Venous invasion	3	16.7
Perineural invasion	1	5.6
Adjuvant therapy	5	27.8
Follow-up (months)	14 (11–30)	
Recurrence (systemic)	3	16.7

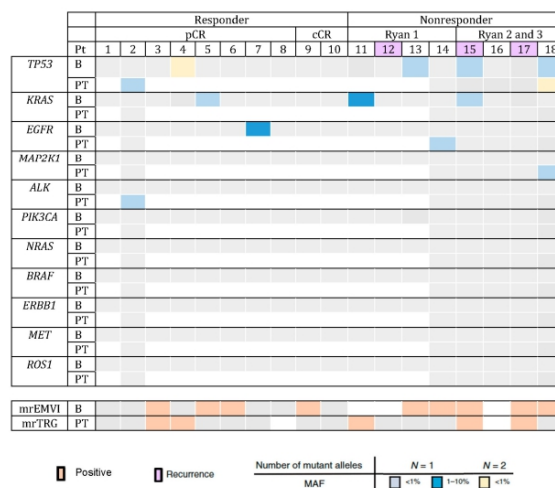
CEA, carcinoembryonic antigen; mrEMVI, MRI detected extramural vascular invasion; nCRT, neoadjuvant chemoradiotherapy; SCRT, short-course radiotherapy; TNT, total neoadjuvant therapy; MRI, magnetic resonance imaging; mrTRG, MRI tumor regression grade; APR, abdomino-perineal resection. Continuous variables presented as median (minimum–maximum range).

2.3. Detection of Somatic Mutations in Plasma

A total of 24 plasma samples from 18 patients were analyzed by NGS. One or more somatic mutations (mutant alleles) were detected in seven (38.9%) patients at baseline and in four out of six (66.7%) patients after preoperative treatment. One or more somatic mutations in TP53, KRAS and EGFR genes were detected in four (22.2%), three (16.7%) and one (5.5%) patient at baseline, respectively. In the post-treatment analysis, we found mutations of TP53 in three patients and mutations in EGFR, MAP2K1 and ALK in one patient each. Two post-treatment mutations were acquired, not detectable at baseline, and in one patient, a TP53 mutation present at post-treatment samples was already present at baseline. These results are shown in Figure 2. The distribution of baseline mrEMVI and post-treatment MRI reevaluation results are also shown in this Figure.

Patients were classified into four groups, as shown in Figure 2: pCR ($n = 8$), cCR ($n = 2$), Ryan 1 ($n = 4$) and Ryan 2 and 3 ($n = 4$).

In the pCR group, one patient had TP53 and ALK mutations with MAF < 1% that appeared after treatment (the only patient of the pCR group with post-treatment samples collected), one patient had two mutations of TP53 with a MAF < 1% before treatment, one patient had a KRAS mutation with MAF < 1% and one patient had an EGFR mutation with MAF between 1 and 10%. No mutations were found in the other patients, as no mutations were found for the cCR group, also with no samples collected post-treatment.



Pt, patient; pCR, pathological complete response; cCR, clinical complete response; B, baseline; PT, post-treatment; mrEMVI, MRI extramural venous invasion; mrTRG, MRI tumor regression grade; MAF, mutant allele fraction. Blank spaces, analysis not conducted or data not available.

Figure 2. Genomic landscape of the mutations detected in plasma and imaging data.

For non-responders, in the Ryan 1 group, only one patient had both samples collected, and in this patient, a new mutation in EGFR appeared after treatment. In one patient a KRAS mutation with a MAF between 1 and 10% was found at baseline, and in another patient a TP53 mutation with MAF < 1% was found. For the Ryan 2 and 3 group, both samples were collected in the four patients selected. One patient had TP53 and KRAS mutations at baseline with MAF < 1%, and another patient presented with a TP53 mutation with MAF < 1% at baseline and with two mutations in TP53 and one mutation in MAP2K1 with MAF < 1% after treatment. In the other two patients, no mutations were found.

2.4. Association between Response to Preoperative Therapy and ctDNA

As shown in Table 2, in our sample nonresponders and poor responders had a higher frequency of positive baseline ctDNA and a lower frequency of post-treatment ctDNA, although no significant association was observed. The MAF distribution is presented in Figure 3, where we can observe that most patients at baseline presented a MAF < 1% and in the six patients with two blood samples collected (pre- and post-treatment), MAF has non-significantly (Wilcoxon test, $p = 0.109$) increased between the two timepoints.

In Figure 2, we showed that all nonresponders with mrEMVI available were mrEMVI positive, whereas for responders this variable is more scattered. In what concerns mrTRG, we observed that non imagiological responders (mrTRG3-4) were also scattered across the groups (responders vs. nonresponders).

In Table 2, we showed that baseline mrEMVI could discriminate nonresponses from complete responders ($p = 0.044$). We then created a variable combining patients with either positive mrEMVI or positive baseline ctDNA and found that this variable was associated with a higher risk of poor response. Due to our small sample, a multivariable logistic regression was not attained. No other parameter could predict response, namely cTN stage, neoadjuvant therapy regimen, interval from therapy to surgery or mrTRG. Furthermore, in additional analysis not presented in the tables, ctDNA was not significantly associated with any of the other clinico-pathological features of the patients.

Table 2. Summary of the relationship between risk factors and main outcomes.

	Nonresponders vs. pCR + cCR	<i>p</i>	Poor Responders vs. Good Responders	<i>p</i>
cTN III	85.7% vs. 80.0%	1.000 ^a	66.7% vs. 85.7%	0.465 ^a
mrEMVI+	100% vs. 40.0%	0.044^a	100% vs. 50.0%	0.229 ^a
NAC vs. nCRT	37.5% vs. 30.0%	1.000 ^a	50.0% vs. 28.6%	0.569 ^a
Interval therapy to surgery (weeks)	11 vs. 9.5	0.161 ^b	11 vs. 14	0.684 ^b
mrTRG 3-5	42.9% vs. 22.2%	0.596 ^a	66.7% vs. 23.1%	0.214 ^a
Baseline ctDNA+	50.0% vs. 30.0%	0.630 ^a	50% vs. 35.7%	1.000 ^a
Baseline MAF	0.044 vs. 0.0	0.479 ^b	0.044 vs. 0.0	0.904 ^b
Either mrEMVI or ctDNA+	2.000 (0.260–15.381)	0.289 ^c	1.667 (0.135–20.578)	0.021^c
Post-treatment ctDNA+	60.0% vs. 100%	1.000 ^a	50.0% vs. 100%	0.467 ^a
Post-treatment MAF	0.057 vs. NA	0.776 ^a	0.159 vs. 0.105	1.000 ^a

pCR, pathological complete response; cCR, clinical complete response; cTN, clinical tumor node stage; mrEMVI, MRI extramural venous invasion; NAC, neoadjuvant chemotherapy; nCRT, neoadjuvant chemoradiotherapy; mrTRG, MRI tumor regression grade; ctDNA, circulating tumor DNA; MAF, mutant allele fraction; ^a Fisher exact test; ^b Mann–Whitney test; ^c McNemar test.

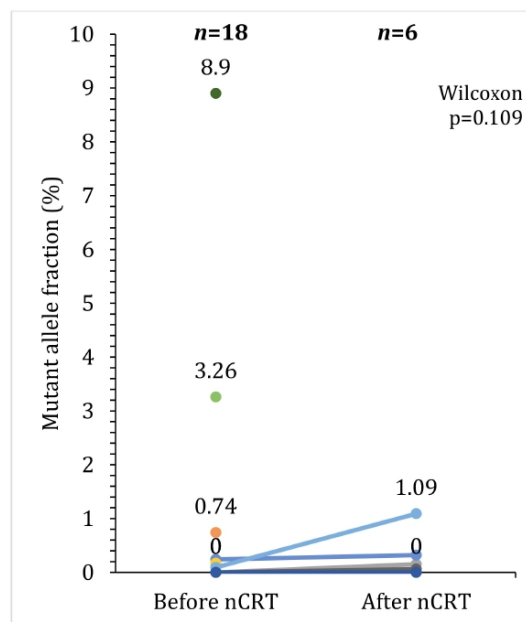


Figure 3. Mutant allele fractions in baseline and post-treatment samples and changes between the two timepoints.

2.5. Association between Disease-Free Survival and ctDNA

We also evaluated the value of ctDNA in disease-free survival. With a median follow-up time of 14 (11–30) months, no patient died and a total of three (16.7%) recurrences, as lung metastases, were registered at a median of 12 months. As seen in Figure 2, patients

with numbers 12, 15 and 17 presented with recurrence and were nonresponders. They all were mrEMVI positive and two of them were imagiological poor responders (mrTRG 3-5). In two of them, no mutations were found; in the other patient, two mutations were found (*TP53* and *KRAS*).

Kaplan–Meier curves, shown in Figure 4, revealed differences in DFS between responders and nonresponders ($p = 0.004$), good responders and poor responders ($p = 0.007$), and clinical stages ($p = 0.014$). We registered no impact of pathological stage on prognosis. No difference in DFS was found for positive baseline or post-treatment ctDNA vs. negative. However, when we accounted for the number of mutations present, those patients with two mutations vs. fewer than two mutations had a worse DFS ($p = 0.005$). No other clinico-pathological factors studied had an impact on prognosis; we did not proceed to search for independent factors of prognosis due to the size of our sample.

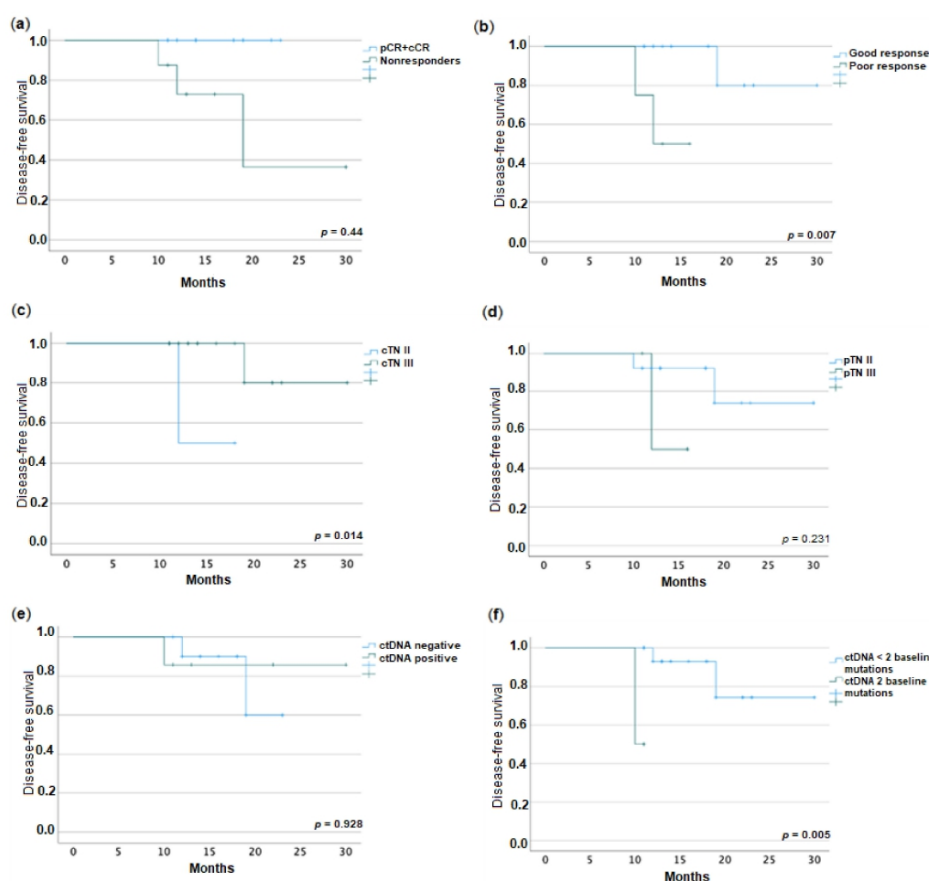


Figure 4. Kaplan–Meier curves of disease-free survival. (a) Responders vs. nonresponders; (b) Good response vs. poor response; (c) Clinical TN stage II vs. III; (d) Pathological TN stage II vs. III; (e) ctDNA negative vs. positive; (f) ctDNA baseline mutations <2 vs. ≥2.

3. Discussion

Several studies have sought to identify clinically useful predictors of pCR after pre-operative therapy for patients with LARC [27]. Some parameters have been reported as possible predictors, including baseline CEA levels, distance of the tumor from the anal verge, tumor size, clinical lymph node metastasis and interval between treatment and surgery [27–29]. Habr-Gama et al., suggested that strict clinical and endoscopic findings of patients with cCR could be useful to select patients for “watch and wait” strategies [30]. However, endoscopic evaluation or MRI evaluation for the response after treatment have low accuracy for pCR [13], with a sensitivity of only about 25% [31]. Furthermore, it has been reported that local regrowth after cCR was about 25% to 50% [32,33], whereas after pCR it was only about 3% [9], with a higher rate of distant metastases after local regrowth (36% vs. 1%) [34]. These data call for an improvement of the methods currently used for predicting pCR.

The systemic biomarker ctDNA is readily available through liquid biopsy and has been shown to detect micrometastasis or minimal residual disease earlier than imaging [35]. Even though some studies reported an association between survival outcomes and ctDNA [17,19,36–40], fewer studies have shown an association with pCR [37,41]. In our study, we intended to understand whether baseline ctDNA (evaluated by NGS available in our laboratory) could help predict response to nCRT in LARC patients. Although we had a small sample limited by budget, we opted to also study the available post-treatment samples, as Murahashi et al. ($n = 85$) had previously shown that dynamic changes in ctDNA performed better than baseline ctDNA in predicting response [37]. The baseline ctDNA was positive in 38.9% of cases, and this rate varies largely in the literature, going from 15 to 77% [42]. Our results showed that baseline ctDNA was not associated with pCR or good response as previously found in other works [36,37]. However, nonresponders or poor responders most frequently had positive baseline ctDNA versus complete or good responders, which shows a tendency already observed in previous studies [38,43]. The lack of correlation between baseline ctDNA and pathological response might reflect the heterogeneity of sensitivity from the tumor to neoadjuvant treatment. Unlike Pazdirek et al. [40] and Wang et al. [33], in which ctDNA was strongly eliminated from plasma from baseline to post-treatment samples (probably due to tumor shrinkage and less ctDNA available from enzymatic digestion induced by radiation damage [44]), in our study we observed that some mutations appeared in the post-treatment sample that were not present initially, although the MAF did not change significantly and could not predict outcomes. This was probably related to the low number of post-treatment analyses or the limited sensitivity of NGS for identifying pCR, as reported by Roesel et al. [45].

The most common genes mutated in our sample were *TP53* and *KRAS*. *TP53* mutation is observed in 50–75% of CRCs. *p53* plays a crucial role in regulating DNA repair and apoptosis in response to radiation, and *TP53* mutations are reported to decrease radiation-induced apoptosis in several cancers [46]. Tumors with *TP53* mutations tend to accumulate through neoadjuvant treatment [47]. This suggests that chemoradiation therapy provides a selective pressure for the expansion of *TP53*-mutant cells in residual tumors [47]; as could happen with other mutations that appeared later in our sample. A meta-analysis of 1830 cases with low wild-type *TP53* status has shown an association with pathological response in LARC patients [48]. Although we could not demonstrate which mutation was more impactful for a good response, *TP53* mutation was the most common mutation among our patients. Furthermore, in the literature miR-34a has been reported as one of the most relevant downstream effectors of *p53*, which blocks the IL-6R/STAT3/miR-34 feedback loop resulting in inhibition of tumor progression in rectal cancer [49]. miR-34a might therefore represent another important biomarker and therapeutic target in rectal cancer.

Also, *KRAS* mutation, the second most common in our sample, belongs to the family of genes most frequently present in human cancers [50]. *KRAS* leads to an epidermal-growth-factor-receptor-independent disturbance of the RAS/RAF/MAPK pathway, which regulates proliferation and survival in rectal cancer [51]. *KRAS* mutation and combined

KRAS/TP53 mutations have been previously independently associated with poor responses after neoadjuvant treatment [52,53], although other reports found no correlation with response [54]. Furthermore, *KRAS* mutations have been associated with a shorter survival in patients with MSI-low tumors. Therefore, the screening for defective DNA mismatch repair through immunohistochemistry (IHC) and/or microsatellite instability (MSI) tests should be performed, although there are still challenges due to the heterogeneity of MSI testing [55].

Previous studies have found an additional benefit for combining imaging features, such as mrTRG and ctDNA, in the prediction of response [33,56]. In our analysis, although mrTRG and none of the other clinical factors were associated with response or ctDNA, we found that baseline positive mrEMVI could predict nonresponders. Also, after combining baseline positive ctDNA and mrEMVI, when at least one of these variables were present the risk of poor response increased. An association between ctDNA and mrEMVI has been observed before in Zhou et al.'s work [38]. This could raise our awareness of the fact that not one but many factors might have to be taken into account to predict response, as many pathways might be involved in the response to chemotherapy. As an indicator that can be assessed by radiologists, although still with a variable inter-rate kappa reliability, the clinical value of mrEMVI and the potential clinical significance of its correlation with ctDNA in predicting response deserve further investigation.

With a median of 14 months of follow-up, tumor progression with distant metastases was registered in 3 out of 18 patients. As previously demonstrated in the literature [57] and also in our study, complete and good responses to therapy improved disease-free survival. Interestingly, patients with cTN II had a worse DFS than those with cTN III. This could possibly be explained by Kim et al.'s [58] so-called “survival paradox”, in which the authors attributed a hypothetical higher biological aggressiveness to pT4N0 colon carcinomas. Besides these factors, no other clinico-pathological factors, such as distance to anal verge, mrEMVI, mrTGR, CEA, pTN, mucinous histology, or vascular or perineural invasion were associated with DFS. As for the marker of study, baseline ctDNA or MAF did not predict recurrence, as seen before [38]. When we studied a small group of patients harboring two mutations instead of one, these could differentiate a group with worse DFS. However, Ji et al. [59] found that lower tumor mutation burden at baseline was associated with worse DFS, for these tumors with higher burden might have a higher immune activity and be more immunogenic, which leads to greater tumor shrinkage. Nevertheless, more work is still needed to understand if different signatures might be associated with different outcomes. Also, the dynamic surveillance of ctDNA throughout treatment has shown to be a more reliable method to predict a higher risk of recurrence [26], which was not possible in this study. The exploration of this method could help clinicians direct the high-risk patients to more intensified chemotherapy regimens.

Our study has several limitations. First, due to the small number of recruited patients and samples (due to logistic problems and non-acceptance by some patients) and the short follow-up period, there were too few events to correct for potential confounding factors and to give enough power to ctDNA to come up as independent factor of pCR and disease-free survival. Second, patients underwent different preoperative therapy regimens based on different risk profiles. Third, tumor biopsy sequencing was not performed in our study. Nevertheless, some mutations detected on ctDNA might not be detected in tumor biopsies, which reflects the tumor evolution and heterogeneity not captured in small biopsy samples. Furthermore, the proportion of patients with positive ctDNA at baseline was low, which could be due to false-negative results related to quantitative (insufficient MAF content) rather than qualitative (lack of altered genes in the NGS panel) reasons. Technical improvement in the gene panel could perhaps increase the detection accuracy. A strength of this study relies on the fact that all samples were collected prospectively, which increases quality of samples and provides more reliable results.

4. Materials and Methods

4.1. Patients

This study prospectively enrolled a pilot cohort of patients with LARC from three Iberian centers (Pedro Hispano Hospital, Matosinhos; Centro Hospitalar Universitário de São João, Porto; and Hospital La Paz, Madrid) between January 2020 and December 2021. All patients were diagnosed with cT3-4 or N+ rectal adenocarcinoma (within 12 cm from anal verge) using the AJCC 8th edition TNM classification [60]. Inclusion criteria included age above 18, treatment with neoadjuvant therapy followed by radical surgery, no metastasis at diagnosis or previous cancers, Eastern Cooperative Oncology Group performance status of 0–2 and written informed consent to participate in the study for sample collection, gene sequencing and data analysis. Patients proposed to the “watch and wait” strategy were also included. This study was approved by the Institutional Review Boards of all Hospitals and was registered at ClinicalTrials.gov (NCT04319354).

The following clinico-pathological data were collected prospectively: age, sex, tumor location from anal verge, clinical stage, mrEMVI, neoadjuvant treatment regimen, MRI response, type of surgery, pathological stage, mucinous histology and venous and perineural invasions.

4.2. Treatment

Patients received either one of two neoadjuvant treatment regimens, according to multidisciplinary team decision, taking into account patient and tumor features and point in time of treatment proposal. Mostly in the beginning of the series, the treatment consisted of standard neoadjuvant chemoradiotherapy (nCRT) comprising a total dose of 50.4 Grey over 5 weeks and concurrent capecitabine or 5-fluorouracil. The second treatment consisted of short-course radiotherapy (SCRT), with 25 Gy administered over 1 week and followed by neoadjuvant chemotherapy with combinations of 5-fluorouracil and folinic acid or capecitabine, plus oxaliplatin (TNT). After completing all neoadjuvant treatments, all patients were re-staged by rectal MRI and then proposed to surgery or the “watch and wait” approach.

4.3. Response Assessment

MRI assessment of tumor regression grade (mrTRG) was evaluated according to Mercury criteria by the radiologists [61]. mrTRG 1 was categorized as “complete regression”, mrTRG 2 as “good regression” and mrTRG 3 to 4 as “poor regression”.

pCR was defined as ypT0N0M0. Pathological response was assessed by the Ryan TRG system [62] as follows: Ryan 1, no viable cells or single cells or small groups of residual cancer cells; Ryan 2, residual cancer outgrown by fibrosis; and Ryan 3, significant fibrosis outgrown by cancer or no fibrosis with extensive residual cancer. Clinical complete response (cCR), a surrogate of pCR, was defined as ycT0N0M0, evaluated by clinical examination (digital rectal examination, endoscopy and MRI) in patients with no disease progression after the 12-month follow-up. Furthermore, for the analysis, the following groups were created: “responders” (cCR + pCR) vs. “nonresponders” (the remaining) and “good responders” (cCR + pCR + Ryan 1) vs. “poor responders” (Ryan 2 + Ryan 3). At the end of patient recruitment and after knowledge of these outcomes, the collected samples were then selected for further analysis of ctDNA.

4.4. Blood Sampling, Cell-Free DNA Isolation and Sequencing

Serum carcinoembryonic antigen (CEA) level was measured at baseline. For ctDNA analysis, blood samples were collected 1–2 weeks before initiation of preoperative therapy (baseline) and after treatment (just before surgery) for some patients. Blood samples were collected into BD Vacutainer®PPT™ plasma preparation tubes (2 × 5 mL) (BD Biosciences, Franklin Lakes, NJ, USA). Plasma was extracted by centrifugation at 1100 rcf for 10 min at room temperature and stored at −20 °C according to laboratory protocol. The samples were purified using PromegaInstrument Maxwell® RSC (Promega Corporation, Madison,

WI, USA). Libraries from cfDNA were prepared with Oncomine Lung cfDNA Assay (Thermo Fisher Scientific, Waltham, MA, USA), available in the laboratory for the most common mutations in rectal cancer. The cfDNA panel used in this study covered 11 genes (*ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1* and *TP53*), with more than 150 hotspots (SNVs and short indels). We tracked the mutations with the highest mutation allele frequency (MAF) in each patient [63]. Patients with detectable mutations were categorized “ctDNA positive”, and the ones with undetectable mutations were characterized as “ctDNA negative”.

4.5. Statistical Analysis

Statistics were performed using IBM SPSS Statistics 28.0.10 (IBM, Armonk, NY, USA). Fisher’s exact test, McNemar test and Mann–Whitney tests were used to compare the risk factors in the study with the main treatment response outcomes. The Wilcoxon test was used to compare median MAF between pre-and post-treatment samples. Survival analysis was conducted through the Kaplan–Meier log rank method to analyze the importance of ctDNA and other factors in disease-free survival (DFS), which was defined as time without recurrence since diagnosis until last follow-up. Values with $p < 0.05$ were considered statistically significant.

5. Conclusions

In conclusion, although these results should be read carefully due to sample size, this study suggests that baseline ctDNA combined with mrEMVI could potentially help predict response to nCRT in LARC patients. Additionally, baseline ctDNA number of mutations might allow the discrimination of groups with different disease-free survival times. Further studies with more patients, samples, timepoints and clinical data integrated with a large number of cancer-related genes are needed to clarify the role of ctDNA as an independent tool in the selection and management of LARC patients. The authors believe that the integration of the data from this study with data coming from other studies in future meta-analyses will likely be useful to provide a better guide for clinical practice by healthcare professionals.

Author Contributions: M.M., J.C.M. and S.C. designed the study. M.M., T.F., D.M.-P. and I.P. performed sample and data acquisition. A.T.V., A.L.D. and P.L. revised imaging data. L.C. performed sample processing and analysis. M.M. analyzed the data. J.C.M. and S.C. verified the data. M.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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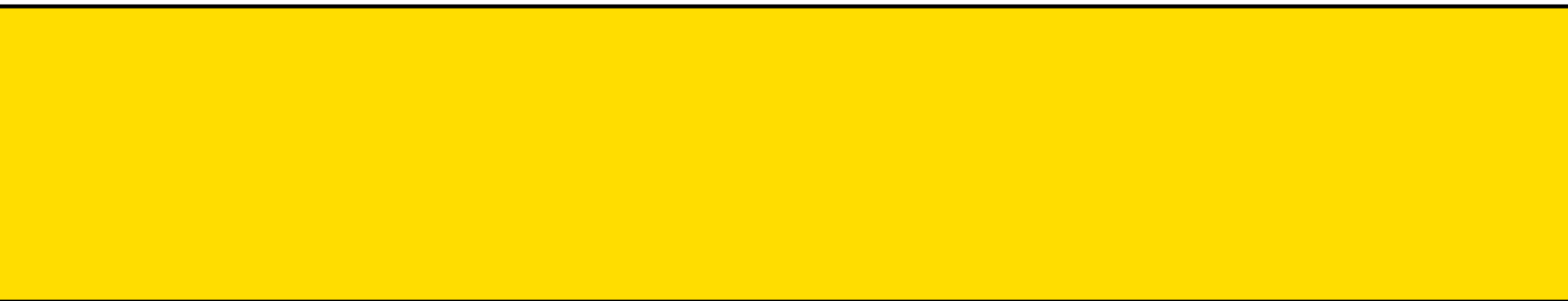
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DISCUSSION



Discussion

The recent advances in the management of LARC patients, with new strategies and oncological regimens, have improved local control in these patients²⁹². However, many patients still present distant disease recurrence and cancer-specific mortality²⁹³. Presently, few clinical factors can predict long-term outcomes and allow a personalized therapy decision making. TNM stage is used as the main tool for decision. Nevertheless, as seen before, a substantial heterogeneity of outcomes happen inside the same stage^{53 293}. Only three biomarkers, MMR-status, RAS-status and BRAF-status are currently available in clinical setting to aid in determining the prognosis of a patient and predicting treatment therapy. As for response to neoadjuvant treatment, there are no clinical or molecular markers that can accurately predict response²⁰, and with the growing choice of organ-sparing approaches, there is a real risk of tumor regrowth of approximately 30%¹⁰⁹. The IWWD (International Watch and Wait Database), a large-scale registry from dedicated centers to the study of “watch and wait” strategy is providing a wider perspective of the outcomes and protocols that should be followed for good results²⁹⁴.

As part of the MDT group meetings, the surgeon must understand these premises, for evermore the best therapy decision for each patient might not include surgery. The decisions should be the result of a discussion between several specialties, including surgeons, oncologists, radiotherapists, gastroenterologists, pathologists, radiologists, psychologists, nutritionists, palliative care doctors, and so on. Furthermore, this discussion should be supported by the knowledge of the latest evidence-based literature and guidelines. With the expanding of therapeutic strategies in the realm of LARC patients, new biomarkers must be studied in order to support those choices.

The research studies that integrate this thesis have focused on the potential utility of circulating biomarkers in supporting those choices. We intended to validate some findings of the literature related to the impact of circulating inflammatory markers and ctDNA in the prevision of pathological response to nCRT

DISCUSSION

and of prognosis in LARC patients. Also, we proposed to show a wide perspective of current studies showing the implication of the use of ctDNA in response to nCRT and prognosis in LARC patients. Subsequently, we provide a discussion and summarization of our findings, and also postulate some premises for the execution of future studies.

1. PN Index and PLR Play a Role in Predicting Response to nCRT in LARC Patients

The host response is becoming increasingly recognized to play an impactful role in clinical outcomes (long-term survival) ²⁹⁵ and in the potential prediction of response to nCRT in LARC ²⁹⁶. A recent meta-analysis for the study of predictive factors of pathological response established that there is currently a lack of robust clinical or radiological biomarkers for predicting such a response ²⁹⁷.

Blood hematological and biochemical biomarkers are inexpensive and readily available in routine clinical practice. Therefore, the first step of our project was to study the impact of circulating markers, to evaluate if there were markers accurate enough to determine response to nCRT. These biomarkers included hemoglobin, mean corpuscular volume, white blood cell count, absolute counts of neutrophils, lymphocytes, monocytes, platelets, albumin, CEA, NLR, PLR, LMR, SII (systemic inflammation index), PNI (prognostic nutritional index) and PN (platelet-neutrophil) index. In a retrospective analysis of 191 patients, from two centers, we achieved a pCR of 12.6% and a Ryan TRG good response of 46.6%. We demonstrated that patients who achieved a poor response to therapy had high PN index (platelet count higher than $350 \times 10^9/L$ and neutrophil count higher than $7.5 \times 10^9/L$) at baseline, cT stage >2 and tumor length over 5 cm. Unfortunately, no conclusion could be made regarding the association of inflammatory circulating markers and pCR, probably due to its low rate in our study. We did not identify any other significant association between circulating biomarkers and response to therapy. This could relate to the relatively small sample size or other confounding factors, but we also pointed out the potential impact of publication bias favoring positive results in other studies of this nature, present in the literature.

In a more recent study, we extended this sample to 245 patients and achieved a pCR of 14.7%. In this sample, male gender, higher tumor length and post-nCRT PLR were independent predictors of pCR. Although PLR had only a moderate sensitivity and specificity for predicting pCR, these results show that inflammatory markers could have an important role in the definition of treatment strategies in the future.

Few studies regarding the value of inflammatory markers, like NLR, have been published since 2014, highlighting the recent interest in this area. A systematic review from 2021 ⁴, reports that studies evaluating the impact of NLR on tumor response to nCRT yielded conflicting outcomes. The negative results could be explained by the use of NLR survival cut-offs and the use of historical values from previous studies (these ranged from 2.77 to 5). Furthermore, in a meta-analysis it was found that none of the authors specifically addressed the prediction of pCR, instead focusing on good responders ¹⁸⁸.

Policicchio et al. was the first to show that PN index at the time of diagnosis had predictive value ($p = 0.039$) in 112 patients with respect to pCR ²⁹⁸. In our first results, although PN index could not predict pCR, it allowed prediction of good responders. Furthermore, the association between PLR and pCR has been previously observed, in two studies in which worse pathological response was linked to high pre-nCRT platelet levels, changes in platelet levels and post-nCRT PLR ²⁹⁹, and to high intra-nCRT and high PLR change rate ³⁰⁰.

Several individual studies have also shown the impact of other markers in response, either individually or combined. Low LMR ³⁰¹, high PNI ³⁰², CRP ³⁰³, mGPS ³⁰⁴, LCR (lymphocyte-to-C-reactive protein ratio), $N \times M$ value (neutrophil $\times$ monocyte) ³⁰⁵, SII ³⁰⁶ and MCV (mean corpuscular value) ³⁰⁷ have proven to be significant predictors of poor or complete pathological response in rectal cancer patients following nCRT, superior to other scores studied in parallel. These results have not, however been validated by other authors ^{226 308 309}.

These indexes could be helpful in identifying high-risk patients and may help select patients for TNT and “watch and wait” strategies. There is still substantial heterogeneity between studies, as cut-offs for most ratios remain highly variable in the literature and require standardization in larger randomized studies.

2. NLR and PN index Can Predict Survival Outcomes in LARC, Adding Value to TNM Stage

We further evaluated the relevance of blood hematological and biochemical circulating biomarkers in the prediction of survival outcomes in LARC patients. We performed a retrospective analysis of 245 patients, from two centers, and found that besides greater tumor dimension, pathological stage III, vascular invasion, absence of adjuvant CT and higher pre-treatment CEA, higher pre- and post-nCRT neutrophil-to-lymphocyte ratios (NLR) significantly predicted overall survival. As for disease-free survival, it was significantly predicted by greater tumor dimensions, patients submitted to abdominoperineal amputation, pathological stage III, perineural invasion, higher circumferential rectal margin and higher post-nCRT PN index, in our analysis. Furthermore, in patients with pathological stage II, post-nCRT PN index allowed the definition of subgroups of higher risk. This was one of the few studies to investigate and evidence the value of several parameters in different timepoints in prognosis, although some scores studied elsewhere, such as PLR, LMR, SII and PNI, could not predict prognosis in this work. We could not study other important markers, such as CRP, fibrinogen or cytokines, because they were not routinely evaluated in the centers in which this study was performed.

Neutrophils secrete cytokines and chemokines, as well as reactive oxygen and nitrogen species, that play an important role in cancer progression ³¹⁰. Lymphopenia, commonly present in these tumors, might help tumor cells escape immune surveillance and prevent damage from the autoimmune response by cytotoxic T cells ³¹¹. Thus, a high NLR level reflects alterations in the cancer microenvironment that favor cancer initiation and progression. Platelets are also thought to be involved in tumor progression by regulating neo-angiogenesis and by helping circulating tumor cells to escape immune surveillance ^{312 313}. PN index could have an impact in predicting DFS, although this association has not been reported before and deserves further consideration, especially in the definition of risk subgroups. The definition of subgroups of prognosis could potentially alter the treatment strategy of patients with LARC in the future, by intensification of CT, change to TNT or possible introduction of immunotherapies for the higher-risk patients.

DISCUSSION

A meta-analysis from 2021 ⁴, including 31 studies with 7553 patients, has concluded that NLR was a superior indicator of unfavorable OS and DFS in patients undergoing curative resection for rectal cancer. The prognostic value of NLR was superior to PLR and comparable to LMR. A more recent meta-analysis ¹⁰, incorporating some of the same studies achieved the same conclusions, but cautioned that only pre-nCRT and not post-nCRT NLR was associated with prognosis, which could be explained by additional changes in the cells produced by the treatment. A summary of the characteristics of the main studies exploring LARC in these meta-analyses are presented in Table 5. A previous meta-analysis ³¹⁴ had explored the value of systemic inflammatory indexes in CRC, but the differences in treatment strategies and survival rates between colon and rectal cancer might bias conclusion in these more inclusive studies. Meanwhile, a Canadian multicenter study by Dudani and colleagues ³¹⁵, with the largest effect size in both meta-analyses already mentioned, reported that NLR level was not associated with OS, warning that the value of NLR in other smaller sized studies could be the work of confounding factors.

Table 4. Main features of studies assessing the impact of NLR in prognosis of LARC patients (adapted from Hamid et al. ⁴ and Lee et al. ¹⁰)

Author, year	Country	N	Follow-up (months) median (range)	Cut-off for NLR	Multivariate analysis
Carruthers et al., 2012	UK	115	37.1	5	Yes
Kim et al., 2014	Korea	102	39 (3-238)	3	Yes
Shen et al., 2014	China	199	31 (1-84)	2.8	Yes
Nagasaki et al., 2015	Japan	201	51.2 (3.6-120.1)	3	Yes
Lino-Silva et al., 2016	Mexico	175	33.5 (10-75)	3	No
Hodek et al., 2016	Czech R.	173	35 (5.6-76.9)	2.8	No
Jung et al., 2017	Korea	984	48	1.7	Yes
Zhao et al., 2017	China	100	45.5	2.25	Yes
Shen et al., 2017	China	202	45 (3-162)	3	No
Sung et al., 2017	Korea	110	31.1	1.75	No
Ward et al., 2018	USA	146	NR	4.47	Yes
Braun et al., 2019	Germany	220	67	4.06	Yes
Cha et al., 2019	Korea	131	73.7	3	No
Jeon et al., 2019	Korea	140	37	2.66	Yes
Dudani et al., 2019	Canada	1237	71	4	Yes
Kim et al., 2019	Korea	173	75 (5-166)	2	No
Zhang et al., 2019	China	76	NR	2	Yes
Sun et al., 2019	China	317	54	2.9	Yes
Bai et al., 2019	China	104	47 (4-60)	2.2	Yes
Zhang et al. 2020	China	472	57 (20-104)	2.3	Yes
Ergen et al., 2021	Turkey	53	43(9-146)	2.49	No

NR, not reported.

Another recent meta-analysis ³¹⁶ concluded that pretreatment LMR, but not PLR, correlated with tumor response and prognosis after curative-intent surgery for rectal cancer. Several individual studies have also shown the impact of other markers in prognosis. Elevated pretreatment PNI ³⁰², LA (lymphocyte count × albumin level), IPI (inflammation-related prognostic index, measured as the combination of high NLR and hypoalbuminemia) ³¹⁷ and FA score (plasma fibrinogen and serum albumin) ³¹⁸ have proven to be significant predictors of survival in LARC patients, and to be superior to other scores studied in parallel. In contrast, other markers like mGPS, AGR (albumin-globulin ratio), SIS (systemic inflammation score, which is a score of serum albumin level and LMR), CRP and CAR (C-reactive protein-albumin ratio) have only showed significance in studies exploring colon and rectal cancer altogether ³¹⁷.

In the context of prognosis, higher pre- and post-nCRT neutrophil-to-lymphocyte ratios (NLR), higher post-nCRT PN index, and other markers described in the literature could potentially identify high-risk LARC patients, optimize the use of adjuvant therapies or aid the choice of other strategies, such as immunotherapy, in order to achieve maximum therapeutic effectiveness. Larger randomized studies are needed, incorporating several of these indexes in LARC patients separately, to define which scores are the most valuable for prognosis prediction and to define cut-offs for each of them. This study suggests that the contribution of several scores could have impact in the sub-classification of patients from different TNM stages. Therefore, the contribution and interaction of different scores should be analyzed to allow the construction of nomograms, to guide clinical therapeutical decision.

3. ctDNA is a Thriving Predictive Tool for Assessing Response and Prognosis in LARC Patients

In order to allow better sub-classification of TNM stage and complement information already found from blood hematological and biochemical parameters, we moved on with the study of ctDNA, also a readily available, non-invasive, circulating biomarker retrieved in liquid biopsy. As stated before, this marker is also representative of inter- and intra-tumor heterogeneity, unlike tissue biopsy of the tumor. Due to its short half-life, liquid biopsies of ctDNA were considered to provide a more real-time information compared with other diagnostic methods ²³³. As the price of methods for ctDNA detection are decreasing, estimated at 500 US dollars per test ³¹⁹, this is an attractive marker to study for potential future clinical application.

In order to explore what was already known in the literature, we performed a systematic review on the impact of ctDNA in the prediction of response and prognosis of LARC patients. We retrieved 21 studies, of which 17 were full-text articles and 4 were abstracts presented in medical conferences, summarizing information of 1499 patients in total.

Regarding TRG, we found that dynamic changes throughout therapy of increasing longer fragments of cfDNA, median value of Alu 237 repeat and integrity index (ratio of longer cfDNA fragments over the levels of short fragments) were associated with no response from tumors to nCRT. Baseline evaluation of mutations from tissue tumor samples in liquid biopsies, TMB, *TP53* or *APC* mutations (negative association) and *MGMT* (O 6-methyl-guanine-DNA methyltransferase) or *ERCC1* (excision repair cross-complementing) genes methylations (positive association) could also predict TRG. In contrast, baseline cfDNA total levels, dynamic changes in *KRAS* mutations and *NPY* (neuropeptide Y) hypermethylation had no association with TRG.

As for pCR, baseline specific mutations from tissue samples, pre-treatment promotor profiling of cfDNA and dynamic changes (until before surgery) of several common mutations (*TP53*, *KRAS*, *APC*, *PIK3CA*, etc.) allowed prediction of this outcome. Instead, levels of cfDNA, pre- and post-treatment measurement of

KRAS/BRAF mutations and baseline *NPY* hypermethylation showed no association with pCR.

Concerning survival outcomes, baseline cfDNA levels above 75th percentile, the analysis of tissue-specific mutations, the common mutations mentioned above, baseline *NPY* hypermethylation, TMB and persistence of ctDNA in 4 time-points (baseline, dynamic changes through nCRT, post-CRT and post-surgery) predicted survival outcomes. In contrast, total cfDNA levels and detection of *KRAS/BRAF* on specific timepoints showed no association with survival.

This review provided a wide view of the published literature on the potential use of cf/ctDNA as a predictor of outcomes in LARC patients. A wide array of methods and timings for liquid biopsy were used and we found many conflicting findings across studies. Nevertheless, the quantification of cfDNA, the analysis of common mutations in CRC setting and certain hypermethylation, at specific timepoints or rather their dynamic changes, seem promising predictors of outcomes. Regrettably, the most accurate analyses still seem dependent on comparison of mutations with the ones in tissue samples, which wanes the potential ambition of ctDNA of making tissue biopsies obsolete.

After the publication of this systematic review, a few more studies were published on this subject. Wang et al.³²⁰ reported, in 119 patients, that a risk predictive model incorporating both ctDNA (feature at baseline, ctDNA clearance and acquired mutation status throughout nCRT) and mrTRG improved performance in predicting pCR in comparison to individual analysis. Also, patients with detectable CRC driver mutations after nCRT and a high-risk feature in imaging were prone to more recurrences. The GEMCAD 1402 multicentric randomized trial³²¹ observed that detectable ctDNA after nCRT was significantly associated with worse survival outcomes in 180 patients. A randomized trial by Liu et al.³²² of 60 patients came to these same very conclusions. Once again, they added that using personalized assays targeting tumor-informed mutation to complement information from universal panel of genes frequently mutated in CRC and low depth sequencing for copy number alterations may improve the performance of these methods. Finally, in the more recent LiBReCa study of 25 patients³²³, positive ctDNA before surgery could predict poor responders, but no association could be found between disappearance of ctDNA mutations and pCR.

DISCUSSION

In brief, the ideal methods and timings for liquid biopsy require validation, but for the time being, serial analysis of ctDNA at the most opportune timings throughout treatment appears to be the most accurate way to make clinical decisions in the future and to investigate the impact of ctDNA in response to nCRT and prognosis of LARC patients in the meanwhile.

Research is growing exponentially in this area and in Table 5, the main ongoing current trials are briefly presented.

Table 5. Main ongoing trials investigating the role of interventional ctDNA for treatment decision making in LARC patients

Trial Location	Trial design Status	Estimated enrollment	Main characteristics
NCT04842006 Finland (SYNCOPE)	Randomized Recruiting	93	TNT and SCRT vs. standard nCRT Assessment of MRD after surgery and correlation with prognosis
NCT04670588 USA	Prospective Recruiting	30	Determine tumor response based on serum ctDNA serial sampling
NCT03615170 China	Prospective Recruiting	200	Assessment of disease-free survival
AC-TRN 12617001560381 Australia (DYNAMIC RECTAL)	Randomized Recruiting	408	Assessment of reduction of adjuvant chemotherapy with the use of ctDNA
NCT, National Clinical Trial; SCRT, short-course radiotherapy; MRD, microscopic residual disease			

4. Prediction of response through imaging features could be complemented with ctDNA analysis

The value of ctDNA in the literature for prediction of response and prognosis is still conflicting, therefore we intended to add more original data from our population to the records from the systematic review, by conducting a multicentric (3 centers) prospective pilot study on this subject. This protocol was registered in the clinical trials website, with the registration number NCT04319354. We collected and analyzed the samples through a clinically relevant and economically efficient NGS panel for the detection of common rectal cancer gene variants in the ctDNA.

Since 2005, the rise of commercially available massively-parallel bench top sequencers have made targeted sequencing and analysis wide available, both in a research and clinical setting ³²⁴. With NGS, many randomly arrayed DNA molecules on a chip are sequenced and captured in parallel. NGS ranges from small targeted sequencing panels to whole exome or genome sequencing ³²⁵ and is ideal for hypothesis free variant detection. It poses a challenge though, due to the limited amounts of fragmented DNA in plasma and high levels of background noise. Nevertheless, new technologies, such as amplicon-based targeted sequencing methods are being developed to identify the low frequency mutations in plasma ²⁴⁵.

We ensued to determine whether ctDNA could be reliably detected in patients with LARC. Overall, we were able to detect ctDNA in 11/24 (45.8%) samples, and our baseline ctDNA was positive in 38.9%, which is an equivalent sensitivity to previous reports, which go from 15 to 77% ³²⁶. Variants were most frequently detected in both *KRAS* and *TP53*, both of which have been previously hypothesized to impact patient response to nCRT in LARC patients ^{199 201 327}, although individually they could not predict outcomes in this sample. We observed a lack of correlation between baseline ctDNA and pathological response, which might reflect the heterogeneity of sensitivity from the tumor to neoadjuvant treatment, although there was a tendency for non and poor responders to present more frequently with positive baseline ctDNA. Unlike other studies ^{320 328}, in which ctDNA was strongly eliminated from plasma from baseline to post-treatment samples (probably due to tumor shrinkage and less ctDNA available from enzymatic digestion induced by radiation damage), in our study we observed that some

mutations appeared in the post-treatment sample that were not present initially, although the mutation allele frequency (MAF) did not change significantly and could not predict outcomes. Even though we had a low number of post-treatment analysis, these conflicting results surely pushes us to expand the sample number and to seek further confirmations on the reliability of the mutational tumor load as a biomarker in rectal cancer. Furthermore, we could not find an association between baseline ctDNA or MAF and recurrence, but a small group of patients harboring two mutations instead of one has shown a worse DFS, although more work is needed to understand this data. While in the literature the dynamic surveillance of ctDNA throughout treatment has been proved to be a more reliable method of predicting survival ³²⁹, this was not possible to evaluate in this study.

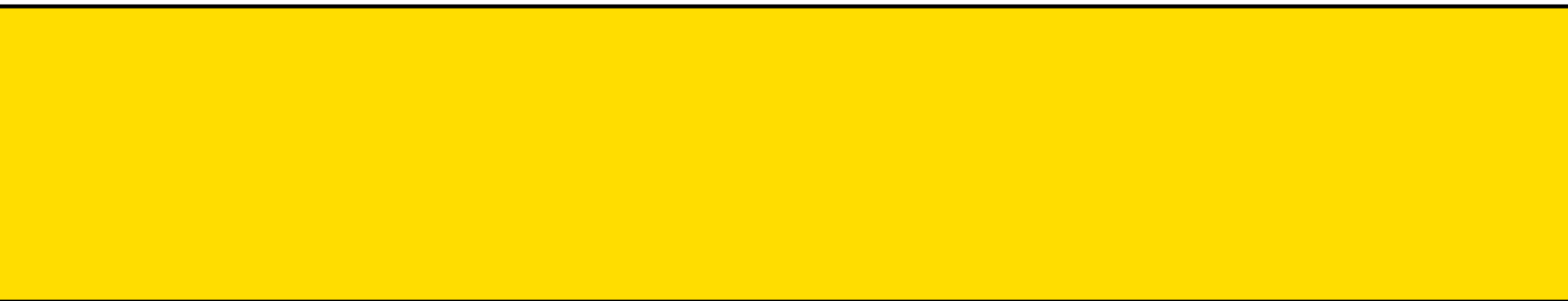
Although there are other reports of the value of the combination of imaging features, such as mrTRG, and ctDNA in the prediction of response ^{320 330}, this factor was not associated with pathological response in our sample. Nevertheless, we found that positive MRI extramural venous invasion (mrEMVI) or the combination of either positive ctDNA or mrEMVI could predict poor response. An association between these two features have been observed before ¹⁹⁷, therefore future studies should look into these associations as other imaging feature beyond mrTRG could increase the sensitivity of prediction of response, and even more if complemented by ctDNA.

Although we were able to make some achievement throughout this work, some setbacks were encountered, which nevertheless contributed to the growth and learning experience as an autonomous researcher by the author of this thesis. This research project was developed in surgical and pathological centers where there were no fully dedicated investigation teams, which made it thoroughly dependent on the good will of professionals. This impacted the study due to the fact that more patients refused to participate, some samples were not collected or were lost in the laboratories. More support given to institutions and departments welcoming researchers would certainly improve the accomplishment of investigation projects. Furthermore, a vast period of patient recruitment occurred during the COVID pandemic, when less cases of rectal cancer were screened and referred to the hospital, which led to a smaller sample than initially estimated. Also, to get a proper sponsorship without the support of an institution proved fairly difficult, which has

limited the amount of sample analysis that could be performed. We also had some limitations related to the laboratory methods, already described in the article 4, but further explained in here. Our detection rate of ctDNA was low compared to the literature ²⁴⁹ and we speculated that this could be due to low plasma volumes leading to low cfDNA concentrations, which can have a detrimental impact in the sensitivity of the NGS analysis ²¹⁷. Also, stored blood samples were used in this study and it is known that prolonged storage of DNA may lead to substantial loss, with annual degradation rates of approximately 7-30% ^{331 332}, reducing even more its detection. Finally, one of the chips used for NGS analysis got damaged, which made us have to repeat the analysis with another chip and lower the budget for additional analyses.

Despite its potentialities, the use of liquid biopsy in routine clinical practice has historically been burdened by considerable costs, heterogeneity in methodology and 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 testing in routine clinical practice. Nevertheless, the cost of high throughput methods has been decreasing ³³³ and the hope given by its proven clinical benefit in other cancers and the growing proofs in LARC, will surely make this field grow exponentially in the next years, until it reaches the clinical setting.

FUTURE PERSPECTIVES



Future Perspectives

Despite the advances in the personalized treatment of rectal cancer, there are still a plethora of molecular alterations to be investigated and establish new molecular response and prognostic biomarkers. As we have shown, inflammatory markers could have a role as biomarkers, adding information to TNM stage in the decision of treatment strategies, including emerging immune checkpoint therapies. Moreover, the dynamic monitoring of ctDNA could be an additional biomarker of prognosis and a valuable complement to imaging methods as a response biomarker.

However, the prediction of outcomes in LARC might not be possible using tumor-specific variants alone. The CRCSC ⁵⁶ have developed a subtyping system based on the gene expression profiling of >4000 patients with CRC. The emergence of novel subtyping systems alert that variable expression profiles might have to be studied in order to predict response and prognosis, and that specific variants alone might not be significant enough. Although their studies provide valuable information for colon cancer patients, information for rectal cancer is still limited, and further testing is needed in a larger number of patients. These will eventually aid in a treatment-stratification for these patients.

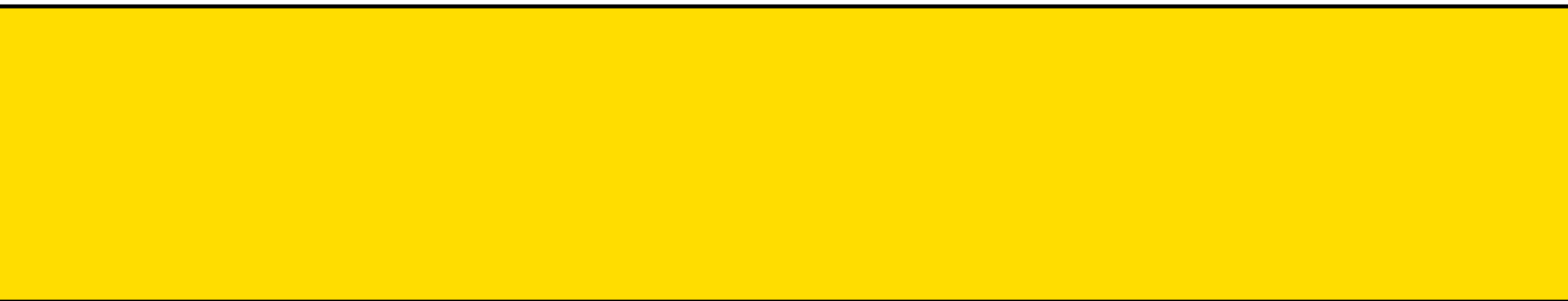
The CMS subtype classification in LARC can be useful in the design of new clinical trials to identify reliable biomarkers for every substage. The future of biomarkers in LARC patients may be a combination of genomic, proteomic and immune factors that contribute to prognostic and predictive scores which will aid the MDT group in designing the patient's individual treatment plan. The inclusion of the markers and mutations studied in this thesis in existing staging systems, will probably help stratify patient risk and ultimately optimize the treatment, survival and quality of life of LARC patients.

As for the continuation of the work stated in this thesis, the first goal is to publish the works already submitted. Then, further funds are required to allow the analysis of the remaining pre- and post-treatment samples and study its association with tumor response, and for analysis of the post-surgical samples to study the potential impact of ctDNA in the prognosis of LARC patients. Also, the maintenance

FUTURE PERSPECTIVES

of prospectively collected databases could provide a more accurate definition of the role of hematological and biochemical parameters in this matter.

CONCLUSION



Conclusion

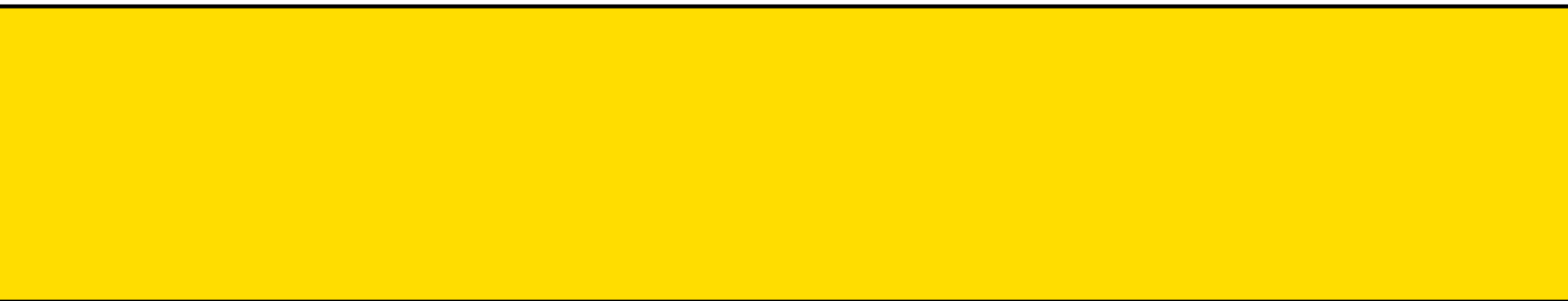
The formulation of scores and nomograms using hematological and biochemical markers already in use in clinical practice could aid in the stratification of high and low-risk patients inside TNM stage. In a population of two centers analyzed retrospectively, we validated that PN index was a marker of poor response to nCRT, PLR was marker of pCR and that NLR and post-nCRT PN index were markers of poor prognosis, allowing the stratification of subgroups of prognosis inside pTNM stage II.

The results of this thesis also suggest that monitoring somatic focal events in ctDNA, a non-invasive biomarker, may aid the stratification and decision of treatment approach of LARC patients. We performed the first systematic review on the role of ctDNA in response to neoadjuvant treatment and prognosis, and observed that in some studies baseline and dynamic monitoring of ctDNA could predict these outcomes. Furthermore, we performed a prospective Iberian multicentric study to validate some of these results and we found that the combination of mrEMVI and baseline ctDNA predicted treatment response and that the number of mutations in ctDNA could predict DFS.

This information could have a significant impact in the treatment of patients with LARC. The benefits of identifying patients who would not respond to therapy could improve quality of life by avoiding unnecessary exposure to cytotoxic agents, or the suggestion of a more aggressive approach to treatment in poor responders and in patients with worse prognosis. Furthermore, the emergence of organ-sparing strategies would likewise benefit from accurate and reliable markers. More work is still required before considering clinical implementation of these methods, from technical standardization to validation in larger translational randomized prospective patient studies.

Nonetheless, this thesis has contributed to the improved understanding of the value of blood parameters and ctDNA in the prediction of nCRT response and prognosis in LARC patients and that these could potentially become useful biomarkers in clinical practice.

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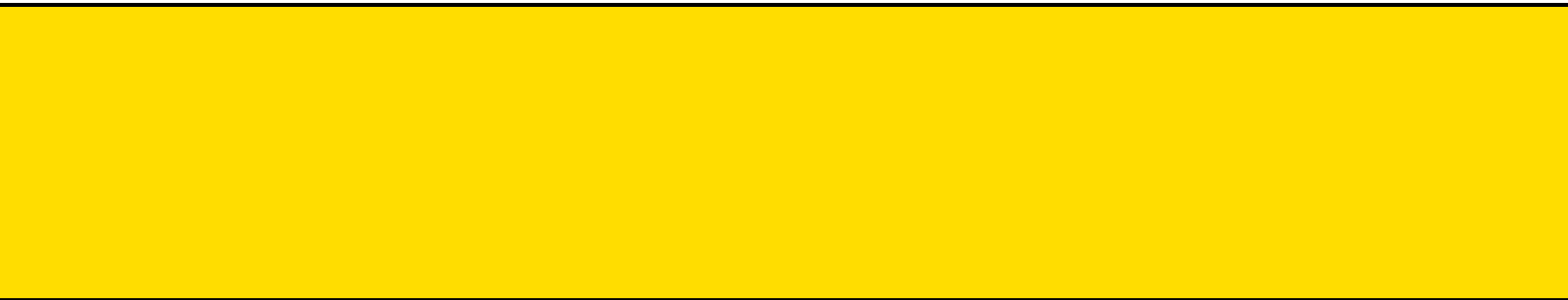
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CURRICULUM VITAE



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Marina Filipa Coelho Morais was born on July 16th, 1987 in Paredes, Portugal. She studied in Penafiel until attending the Faculty of Medicine from Oporto from 2004 to 2010. In the 6th year, she performed basic investigation at the Pharmacology Department, which culminated with the master thesis “Neuropathic pain: evaluation of mechanisms on an experimental model”. After a 1-year general training at Centro Hospitalar do Porto, she started specialization in General Surgery at Centro Hospitalar de São João in 2012. During training, she also visited Memorial Sloan-Kettering Cancer Center, in New York in 2016 (Dr. William Jarnagin and Dr. Julio Garcia-Aguillar). In 2013, she initiated the PhD in Medicine and Molecular Oncology, which she accomplished under the supervision of Professor Silvestre Carneiro and Professor José Carlos Machado.

In April 2018 she obtained the degree of Consultant in General Surgery and maintained functions as Consultant in Colo-Rectal Surgery Unit of Centro Hospitalar de São João. In October 2018, she started working as a Consultant in Hospital Pedro Hispano, in Esophago-Gastric Surgery Unit. In 2018 she visited St Mark's Hospital in London (Dr. Omar Faiz), a hospital that produced much of the current knowledge on Colo-Rectal Surgery. She completed the Clinical Investigator Certification Level 1 from PharmaTrain and the Master in Bariatric Surgery by the University of Madrid (Francisco Vitoria). To this date, she has performed 51 colo-rectal oncological surgeries (of which 16 are rectal) and 50 gastric oncological surgeries. In Hospital Pedro Hispano, she is the tutor of a surgical resident and is also responsible for the residents' training program. She is a member of the Esophago-Gastric and Colo-Rectal Oncological MDT, connecting link of the Commission on Infection and Antimicrobial Resistance Control in the Surgery Department and she was involved on the update of surgical consents of Esophago-Gastric and General Surgery.

She has maintained activity as Assistant Professor in the Department of Surgery and Physiology at Faculty of Medicine of Oporto, since 2014. She published 12 articles in peer-reviewed journals and presented 93 communications in national and international conferences. She is also a reviewer for Porto Biomedical Journal, Revista Portuguesa de Cirurgia and Asian Journal of Reports in Surgery.

LIST OF PUBLICATIONS

- 1- Marina Morais, Telma Fonseca, Diogo Melo-Pinto, Isabel Prieto, Ana Teresa Vilares, Ana Luísa Duarte, Patrícia Leitão, Luís Cirnes, José Carlos Machado, Silvestre Carneiro. Evaluation of ctDNA in the prediction of response to neoadjuvant therapy and prognosis in locally advanced rectal cancer patients: a prospective study. *Pharmaceuticals (Basel)* 2023 Mar;16(3):427. doi: 10.3390/ph16030427.
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- 3- Marina Morais, Telma Fonseca, Raquel Machado-Neves, Mrinalini Honavar, Ana Rita Coelho, Joanne Lopes, Elisabete Barbosa, Emanuel Guerreiro, Silvestre Carneiro. Can pretreatment blood biomarkers predict pathologic responses to neo-CRT in patients with LARC? *Future Oncol.* 2021 Dec;17(35):4947-4957. doi: 10.2217/fon-2021-0707. Epub 2021 Nov.
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- Laparoscopic Splenectomy. *Journal of Laparoendoscopic & Advanced Surgical Techniques*, Aug 2017, Volume 28 (3). doi: 10.1089/lap.2017.0478
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