IDENTIFICAÇÃO E CORRELAÇÃO MOLECULAR E DINÂMICA DE PROTEÍNAS RELACIONADAS À RESISTÊNCIA AO TRATAMENTO NEOADJUVANTE EM CÉLULAS TUMORAIS CIRCULANTES DE PACIENTES COM CÂNCER DE RETO LOCALMENTE AVANÇADO

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RESUMO

Silva VS. **Identificação e correlação molecular e dinâmica de proteínas relacionadas à resistência ao tratamento neoadjuvante em células tumorais circulantes de pacientes com câncer de reto localmente avançado.** [Tese]. São Paulo; Fundação Antônio Prudente; 2021.

INTRODUÇÃO: A quimiorradioterapia neoadjuvante (QRTN) consolidou-se como a principal estratégia para o tratamento do câncer de reto localmente avançado (CRLA). No entanto, respostas heterogêneas são observadas com o tratamento neoadjuvante, com apenas 15-20% dos pacientes com resposta patológica completa (RPC). Diante da necessidade de estratificar os pacientes em respondedores e não respondedores à QRTN antes do seu início, com o objetivo de aprimorar a seleção daqueles com maior probabilidade de obter uma RPC, vários estudos avaliam a identificação de possíveis biomarcadores. O objetivo primário deste estudo prospectivo foi analisar se a ausência da expressão do homólogo B de RAD23 (RAD23B) e da timidilato sintase (TYMS) nas células tumorais circulantes (CTCs) se correlacionaria com a RPC para os pacientes submetidos à QRTN e, assim, identificar possíveis respondedores ao tratamento. Os desfechos secundários foram avaliar a cinética das CTCs antes (C1) e após QRTN (C2), além da correlação da expressão de marcadores de resposta imune, como o Tumor Growth Factor β Receptor I (TGF-βRI) e *Programmed Death ligand*-1 (PD-L1) com a sobrevida livre de doença (SLD) e sobrevida global (SG). **MÉTODOS:** Entre 2016 e 2020, 63 pacientes com CRLA (cT3/T4 e/ou N+) submetidos a QRTN foram incluídos no estudo. As CTCs foram isoladas por ISET® e avaliadas por imunocitoquímica. A expressão de RAD23B, TYMS, PD-L1 e TGF-βRI foi avaliada nesta ordem de prioridade de acordo com o objetivo primário do estudo em cada momento de coleta e a disponibilidade de células (contagem de CTCs > 0) na amostra. **RESULTADOS:** Em C1, RAD23B foi detectado em 54,1% dos pacientes sem RPC e sua ausência em 91,7% dos pacientes com RPC (p = 0,014); Na segunda coleta, dos 13 pacientes com RPC, 10 não apresentaram expressão de RAD23B nas CTCs. Para os pacientes que não obtiveram RPC com QRTN, 51,7% apresentavam a expressão de RAD23B em CTC em C2 (p = 0,06). Na análise univariada (OR =0,077;IC 95%, 0,009-0,661; p = 0,019) e multivariada (OR= 0,064;CI 95%, 0,006-0,75; p = 0,029) para RPC, observamos que a expressão de RAD23B foi associado

com menor chance de resposta em comparação com os pacientes com a ausência da expressão do RAD23B na C1. A ausência da expressão da TYMS foi observado em 90% dos pacientes com RPC e sua expressão em 51,7% sem RPC (p = 0,057). Na avaliação da cinética da CTCs pacientes com CTC2> CTC1 (cinética desfavorável) tiveram pior SLD (p = 0,00025) e SG (p = 0,0036) em comparação com aqueles com CTC2 ≤CTC1 (cinética favorável). A expressão de TGF-βRI em qualquer momento das coletas correlacionou-se com pior SLD (p = 0,059). **CONCLUSÃO:** Demonstramos uma possível correlação entre a ausência de expressão de RAD23B e TYMS nas CTCs com a RPC, sendo um resultado importante para identificar os respondedores ao tratamento neoadjuvante, ajudando individualizar a abordagem terapêutica. Além disso, a cinética desfavorável e a expressão de TGF-βRI nas CTCs se correlacionaram com pior sobrevida.

Descritores: Neoplasias Retais/terapia. Terapia Neoadjuvante/efeitos adversos. Biópsia Líquida. Células Neoplásicas Circulantes

ABSTRACT

Silva VS. **[Molecular and dynamic evaluation of proteins related to resistance to neoadjuvant treatment with chemoradiotherapy in circulating tumor cells of patients with locally advanced rectal cancer].** [Tese]. São Paulo; Fundação Antônio Prudente; 2021.

INTRODUCTION: Neoadjuvant chemoradiotherapy (NCRT) has established itself as the main strategy for the treatment of locally advanced rectal cancer (LARC). However, heterogeneous responses are observed with neoadjuvant treatment, with only 15-20% of patients with complete pathological response (pCR). Given the need to stratify patients into responders and non-responders to NCRT prior to its initiation, in order to improve the selection of those most likely to obtain a pCR, several studies have assessed the identification of potential biomarkers capable of stratifying and monitoring the patient's response. The primary objective of this prospective study was to analyze whether the absence of RAD23 homolog B expression (RAD23B) and thymidylate synthase (TYMS) in circulating tumor cells (CTCs) would correlate with pCR for patients undergoing NCRT and thus identify possible responders to treatment. The secondary outcomes were to evaluate the kinetics of CTCs before (C1) and after NCRT (C2), in addition to the correlation of the expression of immune response markers, such as Tumor Growth Factor β Receptor I (TGF-βRI) and Programmed Death ligand- 1 (PD-L1) with clinical outcomes such as disease-free survival (DFS) and overall survival (OS). **METHODS:** Between 2016 and 2020, 63 patients (pts) with LARC (cT3 / T4 or N +) submitted to NCRT were included in the study. CTCs were isolated by ISET® and evaluated by immunocytochemistry (protein expression). The expression of RAD23B, TYMS, PD-L1 and TGF-βRI was evaluated in this order of priority according to the primary objective of the study at each time of collection (C1, C2 and C3) and the availability of cells (CTC count> 0) in the sample. **RESULTS:** In C1, RAD23B was detected in 54.1% of patients without pCR and its absence in 91.7% of patients with pCR (p = 0.014). In the second collection, of the 13 patients with pCR, 10 did not show RAD23B expression in the CTCs. For patients who did not obtain pCR with NCRT, 51.7% had RAD23B expression in CTC in C2 (p = 0.06). In the univariate (OR = 0.077; 95% CI, 0.009-0.661; p = 0.019) and multivariate (OR = 0.064; 95% CI, 0.006-0.75; p = 0.029) logistic regression

models for pCR, we observed that the expression of RAD23B was associated with a lower chance of response compared to patients with the absence of RAD23B expression in C1. The absence of TYMS expression was observed in 90% of patients with pCR and its expression in 51.7% without pCR (p = 0.057). In the evaluation of CTCs kinetics patients with CTC2> CTC1 (unfavorable kinetics) had worse DFS (p = 0.00025) and overall survival (OS) (p = 0.0036) compared with those with CTC2 ≤CTC1 (favorable kinetics). TGF-βRI expression at any time of the collections was correlated with worse DFS (p = 0.059). **CONCLUSION:** We demonstrated a possible correlation between the absence of RAD23B and TYMS expression in CTCs with pCR, being an important result to identify respondents to neoadjuvant treatment, helping to individualize the therapeutic approach. In addition, the unfavorable kinetics and expression of TGF-βRI in CTCs correlated with worse survival.

Keywords: Rectal Neoplasms/therapy. Neoadjuvant Therapy/adverse effects. Liquid Biopsy. Neoplastic Circulating Cells,

LISTA DE SIGLAS E ABREVIATURAS

5-FU	5-fluorouracil
AJCC	*American joint committee on cancer*
ASCO	*American Society of Clinical Oncology*
CC	Câncer de cólon
CCR	Câncer colorretal
CCRm	Câncer colorretal metastático
CEA	Antígeno Carcinoembrionário
CR	Câncer de reto
CRLA	Câncer de reto localmente avançado
CTCs	Células tumorais circulantes
ctDNA	DNA tumoral circulante
ESMO	*European Society for Medical Oncology*
ETM	Excisão Total do Mesorreto
GLOBOCAN	*Global Cancer Incidence, Mortality and Prevalence*
GRT	Grau de Regressão Tumoral
IC	Intervalo de Confiança
IDH	Índice de Desenvolvimento Humano
IHQ	Imunohistoquímica
ISET	*Isolation by SizE of Tumor cells*
NATs	Neutrófilos Associados ao Tumor
NQRT	Quimiorradioterapia neoadjuvante
OR	Odds Ratio
QRT	Quimiorradioterapia
RAD23B	*RAD23 homolog B*
RCC	Resposta Clínica Completa
RI	Radiação Ionizante
RNM	Ressonância nuclear magnética
RPC	Resposta Patológica Completa

RT	Radioterapia
SLD	Sobrevida Livre de Doença
SG	Sobrevida Global
SLP	Sobrevida livre de progressão
TCLE	Termo de consentimento livre e esclarecido
TGF-β	*Transforming growth factor-β*
TGF-βRI	*Transforming growth factor-β receptor I*
TNT	Tratamento neoadjuvante total
TYMS	*Thymidylate Syntase*
UICC	*Union for International Cancer Control*
WW	*Watch and wait*

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1 INTRODUÇÃO

1.1 EPIDEMIOLOGIA

O câncer colorretal (CCR) é uma doença de importância mundial devido a sua alta incidência e mortalidade. De acordo com dados do GLOBOCAN 2018, o CCR é o terceiro câncer mais incidente e o segundo de maior mortalidade no mundo, enquanto o câncer de reto (CR) é o oitavo mais incidente e juntos compreendem cerca de 11% de todos os cânceres diagnosticados no mundo. No ano de 2018 tivemos mais de dois milhões de novos casos e 1 milhão de mortes relacionadas ao CCR (Bray et al. 2018; Araghi et al. 2019; Sharma 2020).

Os cânceres do reto e da junção retossigmóide são responsáveis por 30% de todos os CCR diagnosticados (Feeney et al. 2019). Determinar a mortalidade especifica para o CR é difícil devido ao grande número de mortes sendo erroneamente classificado como câncer de cólon (Recio-Boiles et al. 2021).

Segundo dados do Instituo Nacional de Câncer-INCA o número de casos novos de CCR estimado para o Brasil para cada ano do triênio de 2020-2022 é de 20.520 casos em homens e de 20.470 em mulheres (Ministério da Saúde 2019). Sem considerar os tumores de pele não melanoma, o CCR em homens é o segundo mais frequente na região Sudeste e Centro-oeste e o terceiro na região Sul. Nas regiões Nordeste e Norte ocupa a quarta posição. Para as mulheres, é o segundo mais frequente nas regiões Sudeste e Sul. Nas regiões Centro-Oeste, Nordeste e Norte, é o terceiro mais frequente.

Dentre os fatores de risco relacionados ao desenvolvimento da neoplasia, estão o tabagismo, sedentarismo, dieta inapropriada, obesidade e ingestão de álcool, mas o maior fator de risco é a idade (Binefa et al. 2014). Características populacionais relacionadas a baixo desenvolvimento sócio-econômico estão associadas à maior incidência de adenocarcinoma colorretal (Doubeni et al. 2012), o que se traduz nas diferenças geográficas encontradas com gradientes distintos entre os níveis do índice de desenvolvimento humano (IDH) (Rawla et al. 2019; Sun et al. 2020).

O CCR é uma doença molecularmente heterogênea, causada pela interação de fatores genéticos e ambientais. As modificações da mucosa colônica são graduais e lentas, duram

aproximadamente de 10 a 15 anos, tempo em que a detecção se faz necessária, antes da progressão de adenoma para carcinoma invasivo (Binefa et al. 2014). Os sintomas mais frequentes do estágio avançado do CCR são: sangramento, desconforto abdominal, mudança do hábito intestinal, perda de peso e anemia. Quanto mais tarde for diagnosticada a doença, pior é o prognóstico (Saha et al. 2002; Hábr-Gama 2005).

Aproximadamente metade dos pacientes diagnosticados com CCR eventualmente desenvolverá metástase. Os principais órgãos envolvidos em metástases são o fígado (75%), pulmões (15%), ossos (5%) e sistema nervoso central (5%). Cerca de 20% dos pacientes são diagnosticados com metástase hepática sincrônica (Vatandoust et al. 2015; Wang et al. 2020).

Tem-se observado uma redução na mortalidade relacionada ao CCR atribuída aos métodos de rastreamento e detecção precoce da doença (Cotterchio et al. 2005; Shaukat et al. 2013), porém são necessários estudos que possibilitem melhoria no tratamento oncológico destes pacientes.

1.2 ANATOMIA E PATOLOGIA

Apesar do fato de o termo CCR ter sido usado por muito tempo para identificar tumores do intestino grosso, evidências crescentes sugerem que o câncer de cólon (CC) e o CR são entidades tumorais distintas. A compreensão das diferenças embrionária, anatômica, microbiota e histopatológica se torna fundamental para o melhor manejo terapêutico dos pacientes (Paschke et al. 2018).

A anatomia do reto é geralmente dividida em três porções. A determinação da localização e os limites anatômicos entre o reto e o sigmóide são importantes para a definição terapêutica. O terço superior do reto é revestido anterior e lateralmente por peritônio, mas é retroperitoneal posteriormente, sem nenhum revestimento de serosa. O terço médio e inferior são completamente extra peritoneais. A ausência de revestimento peritoneal na maior parte do reto é o motivo pelo qual ocorrem maiores taxas de falência locorregional após tratamento cirúrgico do que no CC. O mesorreto é uma camada gordurosa envolvendo o reto e que contém vasos sanguíneos, linfáticos e linfonodos perirretais. A excisão total do mesorreto (ETM) é importante para melhor controle local da doença (Libutti et al. 2011).

Uma determinação precisa da distância do tumor até a borda anal é crucial para a estratificação do paciente, porém com divergências na literatura. A Sociedade Europeia de

Oncologia Médica (ESMO) classifica o CR em terço superior (10-15 cm), médio (5-10 cm) e inferior (<5 cm) (Schmoll et al. 2012). Por outro lado, a União para o Controle Internacional do Câncer (UICC) define diferentes valores de corte em 12-16 cm como alto, 6-12 cm como médio e <6 cm como baixo CR (Attenberger et al. 2020).

A maioria das neoplasias do reto são adenocarcinomas (de 93 a 95%). Carcinoma espinocelular primário do reto é extremamente raro e geralmente é tratado de maneira similar ao carcinoma espinocelular do canal anal (Glimelius et al. 2013).

1.3 ESTADIAMENTO

O estadiamento do CR segue o estadiamento TNM da *American Joint Committee on Cancer* (AJCC) e é realizado com anamnese e exame físico completo, hemograma, função renal e hepática e dosagem sérica do antígeno carcinoembrionário (CEA). Colonoscopia completa é indicada para exclusão de tumores sincrônicos em pacientes não obstruídos. Radiografia de tórax ou preferencialmente tomografia computadorizada de tórax para exclusão de metástase pulmonar, tomografia computadorizada ou ressonância magnética (RNM) de abdome com a finalidade de excluir doença hepática e ou peritoneal. Para avaliação locorregional, a RNM da região pélvica ou o ultrassom endoscópico retal tem maior acurácia para o estadiamento e planejamento terapêutico cirúrgico e pré-operatório (Engelen et al. 2013; Curvo-Semedo 2020).

A RNM da pelve é particularmente útil, pois não só fornece informações sobre o tamanho e a localização do tumor no reto, mas também é útil para identificar o envolvimento potencial de linfonodos, invasão extramural e a extensão da doença circunferencial. Os tumores do estágio T1 estão confinados à submucosa, os tumores do estágio T2 se estendem, mas não além, da muscular própria, os tumores do estágio T3 se estendem além da muscular própria para a gordura mesorretal e os tumores do estágio T4 envolvem órgãos adjacentes ou o peritônio (Beets-Tan et al. 2004; Kaur et al. 2012; Curvo-Semedo 2020).

O PET-CT não é um exame padrão no estadiamento no câncer de reto, porém possui sensibilidade maior na detecção de metástase à distância, especialmente acometimento linfonodal abdominal (Libutti et al. 2011; Glimelius et al. 2013; Kijima et al. 2014).

Os tumores iniciais são aqueles T1N0 ou T2N0, para os quais o tratamento cirúrgico com margens negativas é geralmente curativo, uma vez que as chances de recorrência local

são baixas. Os tumores mais avançados, T3, T4 ou N positivos necessitam de uma abordagem multimodal para melhores resultados (Glimelius et al. 2013).

1.4 TRATAMENTO DO CÂNCER DE RETO

1.4.1 Tratamento cirúrgico

O tratamento cirúrgico é o principal tratamento curativo para o CR. As modalidades cirúrgicas incluem a ressecção transanal que pode ser endoscópica ou cirúrgica e a ressecção abdominoperineal, a depender da extensão e localização da doença (Ptok et al. 2007; Kim et al. 2014). O objetivo da ressecção é a retirada do tumor com margens adequadas, bem como da drenagem linfática e linfonodos regionais. No reto, o mesorreto é a estrutura gordurosa que contém vasos sanguíneos, linfáticos e linfonodos perirretais (Libutti et al. 2011; Delibegovic 2017).

A ETM refere-se à excisão do reto e do tumor em bloco com sua drenagem vascular e linfática e foi estabelecida como o padrão ouro para o tratamento cirúrgico do CR em 1986, quando o Professor RJ Heald descreveu pela primeira vez o procedimento como um meio de remover os depósitos tumorais descontínuos no mesorreto que são mais prováveis de causar falha do tratamento local (Heald et al. 1986).

A ressecção abdomino-peritoneal é um procedimento que remove o reto por inteiro bem como o complexo esfincteriano. Promove baixas taxas de recidiva local, porém resulta em colostomia definitiva (Campelo et al. 2016).

Por fim, o tratamento multimodal pré-operatório associado a melhores técnicas cirúrgicas proporcionaram melhores resultados funcionais pós-operatórios e redução das chances de recidiva (Sun et al. 2020).

1.4.2 Tratamento com quimiorradioterapia no pré-operatório *versus* no pós-operatório

O tratamento de quimiorradioterapia (QRT) inicialmente era realizado no pós-operatório. Em 2004, foi publicado um estudo alemão comparando o tratamento de QRT no pré-operatório com o realizado no pós-operatório (Sauer et al. 2004). Foram randomizados 421 pacientes com adenocarcinoma de reto estádios II ou III para tratamento pré-operatório de QRT e 402 pacientes para tratamento pós-operatório. Em pacientes avaliados antes da neoadjuvância como possíveis candidatos a cirurgia abdomino-perineal pelo cirurgião, a taxa

de cirurgia com preservação de esfíncter foi de 39% no grupo do tratamento pré-operatório e apenas 19% no grupo de tratamento pós-operatório (p= 0.004). O estudo não encontrou diferença no tempo de sobrevida global (SG) entre os grupos estudados. A recorrência local em 5 anos foi significativamente reduzida no tratamento pré-operatório (6% *versus*13%; p=0.006). Embora sem aumento da SG, o estudo demonstrou que resultados de melhor controle local, menor toxicidade aguda e maior índice de preservação de esfíncter favoreceram o emprego do tratamento neoadjuvante no câncer de reto. Após 11 anos, sua atualização demonstra manutenção dos benefícios a favor da neoadjuvância (Sauer et al. 2012).

Outro estudo, o NSABP R-03 (Roh et al. 2009), também comparou o tratamento de QRT neoadjuvante com o adjuvante no tratamento do câncer de reto localmente avançado (CRLA) e demonstrou benefício em sobrevida livre de doença (SLD) (64,7 *versus* 53,4%, p=0,011) a favor dos indivíduos submetidos à neoadjuvância e tendência a maior SG em 5 anos (74,5 *versus* 65,6%, p=0,065), sem significância estatística.

O estudo coreano com 240 pacientes (Park et al. 2011) que comparou o tratamento de QRT pré e pós-operatório (a capecitabina foi a quimioterapia utilizada), demonstrou que não houve diferença em SLD ou de SG. No entanto, para os pacientes com tumores mais distais (< 5 cm da margem anal), o tratamento pré-operatório apresentou maiores taxas de preservação de esfíncter.

1.4.3 Tratamento com radioterapia

A radioterapia (RT) é um pilar importante no tratamento multimodal do CRLA. Em alguns tumores, como os de mama , a RT pode ser administrada como tratamento de longo curso usando fracionamento convencional (1,8–2 Gy / fração) ou como tratamento de curta duração usando hipo-fracionamento (> 2 Gy / fração) (Tseng et al. 2019; Fang M et al 2020).

O estudo STOCKHOLM III comparando longo curso RT (25 x 2 Gy) com cirurgia retardada com o grupo de RT de curta duração (5 x 5 Gy) mostrou resultados oncológicos semelhantes em termos de recorrência local, metástases à distância, sobrevivência e complicações, indicando que esses tratamentos são muito semelhantes (Erlandsson et al. 2017).

Dois ensaios clínicos de fase III (POLISH e TROG 01.04) relataram uma comparação direta entre RT neoadjuvante e QRT, sugerindo que as duas abordagens são amplamente

comparáveis em sua capacidade de reduzir o risco de recorrência local e, portanto, ambas são opções aceitáveis. Nestes estudos, as taxas de recorrência de cinco anos para RT neoadjuvante e QRT foram 27% e 30% respectivamente, enquanto as taxas de SG foram de 74% e 70% (Bujko et al. 2006; Ngan et al. 2012).

1.4.4 Tratamento com quimioterapia concomitante à radioterapia

Em relação ao papel da quimioterapia no tratamento multimodal do CR, o estudo EORTC 22921 (Bosset et al. 2014) analisou o valor da adição da quimioterapia concomitante à radioterapia pré-operatória, bem como da quimioterapia adjuvante. Não houve diferença de SG no grupo de radioterapia pré-operatória isolada em relação ao grupo que realizou quimioterapia e radioterapia. No entanto, o risco de recorrência local foi significantemente menor nos grupos que realizaram quimioterapia. Os pacientes submetidos à QRT pré-operatória apresentaram significativamente maiores taxas de resposta patológica completa (RPC). Na atualização do estudo com seguimento de mais de 10 anos, tem-se que a incidência acumulativa de recorrência local em 10 anos foi de 22,4% no braço da radioterapia isolada comparada a 11 a 15% nos três grupos que receberam quimioterapia. A adição de quimioterapia adjuvante não resultou em aumento de SG em 10 anos ou SLP comparado aos braços que não realizaram quimioterapia adjuvante. Dessa forma, o estudo conclui que há benefício na diminuição da recorrência local com a adição de quimioterapia, no entanto a quimioterapia adjuvante baseada em 5-fluorouracil (5-FU) após tratamento pré-operatório não acrescentou benefício em SG ou de SLD.

Em 2012, foi publicado o trabalho ACCORD 12/0405 PRODIGE 2 comparando dois regimes diferentes de QRT pré-operatória para o CR. Foram incluídos 598 pacientes para tratamento neoadjuvante com capecitabina concomitante a radioterapia com dose de 45 Gy ou capecitabina mais oxaliplatina concomitante a radioterapia com dose de 50 Gy. O objetivo primário foi taxa de RPC. Não houve diferença significativa na taxa de RPC (13,9% *versus* 19,2% para o regime intensificado, p= 0,09). Em três anos de seguimento, não houve diferença na incidência acumulativa de recorrência local (6,1% *versus* 4,4%), SG (87,6 *versus* 88,3%) ou SLD (67,9% *versus* 72,7%). Toxicidades grau 3 ou 4 foram relatadas apenas em 4 pacientes no grupo da capecitabina isolada e em dois pacientes no grupo da combinação com oxaliplatina. Com esses resultados, não houve benefício clinico na intensificação do tratamento neoadjuvante (Gerard et al. 2012).

Em 2013 foi apresentado no congresso da *American Society of Clinical Oncology*-ASCO os dados preliminares do trabalho PETACC-6 (Schmoll et al. 2013), que randomizou 1094 pacientes com câncer de reto estádios cT3, cT4 ou linfonodo positivo para dois braços de tratamento: o primeiro consistia em radioterapia pré-operatória 45 Gy concomitante a capecitabina seguido de capecitabina adjuvante (braço 1); o segundo, o mesmo regime de tratamento com a adição de oxaliplatina ao tratamento pré-operatório e ao tratamento adjuvante (braço 2). Objetivo primário foi SLD. A taxa de RPC foi de 11,3% no braço 1 e 13,3% no braço 2 (p= 0,31). A taxa de ressecção R0 foi de 92% no braço 1 e 86,3% no braço 2. A taxa de preservação de esfíncter também foi semelhante entre os grupos (70% *versus* 65%; p= 0,09). A toxicidade grau 3 ou 4 foi maior no braço 2 em relação ao braço 1 (36,7% *versus* 15,1%). Não foram vistas diferenças nas complicações pós-operatórias. 91% dos pacientes no braço 1 receberam a dose completa ou mais de 90% da dose de quimioterapia prevista, comparada a apenas 63% dos pacientes no braço 2. Os dados de sobrevida foram apresentados em 2014 e mostraram que a SLD em 3 anos foi de 74,5% para o braço 1 e 73,9% para o braço 2. Dessa forma, a adição de oxaliplatina levou a diminuição da adesão ao tratamento, aumento da toxicidade pré-operatória e não melhorou os resultados cirúrgicos ou a SLD (Schmoll et al. 2014).

Ao contrário dos trabalhos acima, o estudo CAO/ARO/AIO-04 sugeriu um benefício em SLD com a adição de oxaliplatina no tratamento neoadjuvante. O estudo incluiu 1265 pacientes com adenocarcinoma de reto, clinicamente estadiados como cT3, cT4 ou linfonodo positivo em dois grupos: grupo controle que consistia em tratamento pré-operatório com radioterapia 50,4 Gy e 5-FU infusional concomitante, seguido de cirurgia e 5-FU infusional adjuvante; e o grupo experimental que consistia em radioterapia pré-operatória 50,4 Gy e 5-FU mais oxaliplatina concomitante, seguido de cirurgia e quimioterapia adjuvante com 5-FU mais oxaliplatina. O objetivo primário foi SLD. Dos resultados alcançados, tem-se que a SLD em 3 anos foi de 75,9% no grupo experimental e 71,2% no grupo controle (HR 0,79; CI 0,64-0,98; p= 0,03). A toxicidade grau 3 ou 4 pré-operatória e pós-operatória ocorreram, respectivamente, em 24% e 36% no grupo experimental e em 20% e 36% no grupo controle. Por este estudo, a adição de oxaliplatina ao tratamento de QRT neoadjuvante e à quimioterapia adjuvante trouxe benefício em SLD (Rödel et al. 2015).

Como visto, o uso de oxaliplatina em combinação com 5-FU no tratamento multimodal do câncer de reto possuia dados conflitantes até as publicações atuais que discutiremos a diante com a abordagem do tratamento neoadjuvante total (TNT).

O Irinotecano é outro quimioterápico com ação comprovada no câncer colorretal metastático (CCRm) e foi estudado em alguns trabalhos de fase II no tratamento do CRLA. Um estudo alemão (Willeke et al. 2007), fase II, de braço único, avaliou a eficácia e segurança do uso de irinotecano semanal em combinação com capecitabina e radioterapia concomitante como tratamento neoadjuvante de CRLA (cT3, cT4 ou linfonodo positivo). Todos obtiveram ressecção R0 e 5 pacientes (15%) obtiveram RPC. A SG em 3 anos foi de 80% para aqueles que foram submetidos a cirurgia.

Já o estudo espanhol, também fase II de braço único, incluiu 74 pacientes portadores de CR com estadiamento cT3 ou cT4 para tratamento neoadjuvante com irinotecano e 5-FU concomitante a radioterapia. Os autores encontraram 13,4% de RPC e 49,3% de *downstaging* na peça cirúrgica (Navarro et al. 2006).

Um estudo fase II randomizado, que avaliou 106 pacientes com câncer de reto distal cT3 ou cT4 em dois braços de tratamento neoadjuvante: o braço 1 consistia em 5-FU infusional concomitante a radioterapia hiperfracionada (duas vezes ao dia) na dose de 45,6 Gy mais um *boost* de 9,6 a 14,4 Gy; braço 2 consistia em 5-FU infusional mais irinotecano semanal concomitante a radioterapia com fracionamento convencional na dose de 45 Gy mais *boost* de 5,4 a 9 Gy. A taxa de RPC foi de 30% no braço 1 e 26% no braço 2. Taxa de recorrência locorregional foi de 16% no braço 1 e 17% no braço 2. A SG em 5 anos foi 61% para o braço 1 e 75% para o braço 2. A SLD foi de 78% e 85 % para o braço 1 e braço 2, respectivamente. Os autores concluem que houve altas taxas de SLD em ambos os braços (Mohiuddin et al. 2013).

O estudo ARISTOTLE de fase 3 recentemente concluído avaliou que a adição do irinotecano ao tratamento neoadjuvante de QRT com capecitabina não melhorou significativamente a taxa de RPC e foi associada a uma diminuição da aderência ao tratamento neoadjuvante devido a uma taxa mais elevada de efeitos adversos (Sebag-Montefiore et al. 2020).

Sendo assim, a combinação de irinotecano ao 5-FU em concomitância à radioterapia neoadjuvante ainda necessita de mais estudos e seu uso ainda não é um padrão.

1.4.5 Quimioterapia neoadjuvante isolada

Uma revisão sistemática e meta-análise, englobando 25 estudos e 6.548 pacientes, procurou determinar o impacto de longo prazo da QRT pré-operatória nas funções anorretal, sexual e urinária. Os resultados mostraram que disfunção anorretal ocorre mais frequentemente após terapia multimodal com QRT e ETM do que após ressecção do mesorreto isolado. O efeito negativo na função urinária e sexual não foi demonstrado na presente meta-análise. Os autores comentam ainda que os trabalhos avaliados na literatura possuem graus de evidência limitados e outros estudos são requeridos para mais informações (Loos et al. 2013).

Dado os efeitos colaterais da radioterapia e à sensibilidade do CR a poliquimioterapia moderna, alguns estudos mais recentes procuraram estabelecer o papel da quimioterapia neoadjuvante sem radioterapia.

O primeiro deles, um estudo fase II, incluiu 32 pacientes estádios II ou III de CR. Todos os pacientes eram candidatos à cirurgia de ressecção anterior com ETM. Os pacientes receberam tratamento neoadjuvante com 6 ciclos de FOLFOX (5-FU, leucovorin e oxaliplatina) e bevacizumabe nos 4 primeiros ciclos (Schrag et al. 2014). Pacientes que apresentassem doença estável ou progredissem ao tratamento, seriam encaminhados para a radioterapia pré-operatória. Aqueles respondedores à quimioterapia eram encaminhados diretamente à cirurgia. O desfecho primário avaliado foi taxa de ressecção R0. Dos 32 participantes, todos (100%) apresentaram ressecção R0. Dois pacientes não completaram quimioterapia pré-operatória devido à toxicidade cardiovascular, ambos foram encaminhados para radioterapia pré-operatória. Dos 30 pacientes que completaram a quimioterapia pré-operatória, todos apresentaram regressão tumoral sem a radioterapia. A taxa de RPC a quimioterapia isolada foi de 25% (8/32). Nenhum paciente apresentou recorrência local em 4 anos. A SLD em 4 anos foi de 84%. Os autores concluem que, em pacientes selecionados, a quimioterapia neoadjuvante e o uso seletivo de radioterapia não comprometem os resultados.

Atualmente, o estudo fase III PROSPECT está em recrutamento para avaliar essa estratégia de tratamento. O ensaio PROSPECT (ClinicalTrials.gov identifier: NCT01515787) foi projetado para determinar se a quimioterapia neoadjuvante com 5-FU e oxaliplatina (FOLFOX) poderia ser usada como uma alternativa à QRT neoadjuvante sem comprometer os resultados do tratamento e poupar esses pacientes de toxicidade excessiva.

Em 2015, foram apresentados no congresso da ASCO os resultados preliminares do estudo FOWARC. Foram randomizados 495 pacientes com CR até 12 cm da margem anal, clinicamente estadiados como estádios II ou III para receberem tratamento neoadjuvante com radioterapia concomitante a 5-FU (braço controle) ou radioterapia com FOLFOX (braço FOLFOX-RT) ou 4 a 6 ciclos de FOLFOX isolado. Taxa de resseção R0 foi, respectivamente, 90,1%; 88,2% e 91,2% no braço controle, braço FOLFOX-RT e braço FOLFOX. A taxa de RPC foi de 12,5%; 31,3%; 7,4%, respectivamente. *Downstaging* cirúrgico foi achado em 34,7%; 57,8% e 37,9%, respectivamente. As maiores toxicidades e complicações pós-operatórias foram encontradas nos pacientes que receberam radioterapia. De acordo com os dados apresentados do trabalho, FOLFOX concomitante com radioterapia resultou nas maiores taxas de RPC, FOLFOX neoadjuvante isolado atingiu *downstaging* semelhante com menor toxicidade comparado com 5-FU concomitante à radioterapia (Deng et al. 2015). Seus resultados finais foram publicados em 2019 e mostraram que quimioterapia com FOLFOX, com ou sem radiação, não melhorou significativamente a SLD em 3 anos comparada com a radioterapia associada com 5-FU em pacientes com CRLA. Nenhuma diferença significativa nos resultados foi encontrada entre mFOLFOX6 sem radioterapia e 5-FU com radioterapia, o que requer uma investigação mais aprofundada do papel da radioterapia nesses regimes (Deng et al. 2019).

1.4.6 Tratamento neoadjuvante total (TNT)

Apesar da diminuição significativa nas taxas de recorrência local de 10% para 5% proporcionada pelo tratamento neoadjuvante com QRT, metástases à distância e morbidade do tratamento ainda são um grande problema. Com isso, o racional do uso de poliquimioterapia neoadjuvante associada antes ou após o tratamento com QRT é bastante atraente, na tentativa de reduzir as taxas de recidiva à distância e aumentar as taxas de RPC (Papaccio et al. 2020).

Conceitualmente o TNT consiste numa estratégia terapêutica em que o tratamento pré-operatório não inclui apenas o tratamento padrão com QRT, mas também vários meses de poliquimioterapia sistêmica (por exemplo, FOLFOX / CAPOX e ou FOLFIRINOX). Um dos principais objetivos de ofertar o tratamento com quimioterapia antes da cirurgia seria aumentar a aderência ao tratamento sistêmico, já que muitos pacientes após a cirurgia não conseguem completar o tratamento adjuvante, sendo talvez uma das justificativas para que os estudos que avaliaram a adjuvância não tenham atingido seus benefícios. Outro ponto a

ser considerado é que ao iniciar o tratamento com quimioterapia estaríamos proporcionando um incremento na taxa de resposta tumoral além do tratamento precoce da doença micrometastática (Tuta et al. 2019; Papaccio et al. 2020).

A segurança do TNT foi demonstrada pela primeira vez no estudo espanhol GCR-3, que randomizou 108 pacientes para QRT de longo prazo seguido de CAPOX e ETM e CAPOX adjuvante ou para indução de CAPOX seguido de QRT e ETM. Neste estudo não houve diferenças nas taxas de falha local de 5 anos (2% vs 5%; p=0,61), falha distante (21% vs 23%; p=0,79), SG (78% *versus* 75%; p=0,64) ou RPC (13% *versus* 14%; p=0,94) (Fernandez-Martos et al. 2015).

Em um estudo retrospectivo com 161 pacientes com CRLA que apresentavam fatores de alto risco para recorrência, 72 pacientes receberam terapia padrão (grupo padrão) e 89 pacientes receberam TNT (grupo TNT). TNT mostrou uma proporção maior de RPC (23% vs 7%; p=0,01), maior T e N-downstaging (70% e 94% *versus* 51% e 86%), ressecção R0 equivalente (95% *versus* 93%), menor tempo para o fechamento do estoma (média: 20 *versus* 33 semanas; p<0,05), maior complacência durante o tratamento com a quimioterapia (completou todos os ciclos 87% *versus* 76%; p<0,05), menor proporção de toxicidade aguda grau ≥ 3 durante a quimioterapia (3% *versus* 14%, p <0,05) (Tuta et al. 2021).

Inicialmente, as primeiras conclusões relevantes à respeito do TNT foram provenientes do estudo retrospectivo do Memorial Sloan Kettering que relata dados de 320 pacientes com CRLA tratados com TNT em comparação com os pacientes tratados com o tratamento neoadjuvante convencional. Maior exposição a quimioterapia foi detectada nos pacientes da coorte do TNT. A taxa de resposta completa, incluindo RPC e clínica em pacientes não submetidos à cirurgia, foi de 36% na coorte TNT em comparação com 21% na coorte do tratamento convencional (Cercek et al. 2018).

Em uma revisão sistemática com 28 estudos (3 retrospectivos e 25 prospectivos com um total de 3.579 pacientes) em que foram incluídos na análise final 2.688 pacientes tratados com TNT e 891 com QRT neoadjuvante, a taxa combinada de RPC foi de 22,4% em todos os pacientes tratados com TNT. Em 10 estudos comparativos com dados disponíveis, os pacientes submetidos a estratégia do TNT apresentaram maiores chances de RPC de 39% (p=0.001) (Petrelli et al. 2020).

Recente metanálise publicada no JAMA em dezembro de 2020 com 7 estudos, incluindo um total de 2.416 pacientes, dos quais 1206 receberam TNT, a prevalência

combinada de RPC foi de 29,9% (variação, 17,2% - 38,5%) no grupo TNT e 14,9% (variação, 4,2% -21,3%) no grupo de QRT convencional. O grupo submetido a estratégia do TNT teve uma maior chance de atingir uma PCR (*odds ratio* [OR], 2,44; intervalo de confiança (IC) de 95%, 1,99-2,98). Não houve nenhuma diferença estatisticamente significativa na proporção de preservação do esfíncter (OR, 1,06; IC de 95%, 0,73-1,54) ou ileostomia (OR, 1,05; IC de 95%, 0,76-1,46) entre os grupos. Apenas três estudos apresentaram dados sobre a SLD, e a análise agrupada mostrou chances significativamente maiores de SLD em pacientes do grupo do TNT (OR, 2,07; IC 95%, 1,20-3,56). Os resultados desta revisão sistemática e meta-análise sugerem que o TNT é uma estratégia promissora no CRLA, com taxas superiores de PCR em comparação com a terapia padrão (Kasi et al. 2020).

Recentemente foram publicados os dados do uso do FOLFIRINOX modificado na estratégia do TNT com o estudo PRODIGE 23. A taxa de PCR foi significativamente maior em pacientes que foram tratados com o TNT (27,5% *versus* 11,7%). Em um acompanhamento médio de cerca de 4 anos, as taxas de SLD em 3 anos foram significativamente maiores no grupo de quimioterapia neoadjuvante (76%) do que no grupo padrão de 69%; (p=0.034), atribuído a uma taxa diminuída de metástases à distância. No entanto, esse benefício não se traduziu em um ganho significativo na SG (p = 0,077) (Conroy et al. 2021). Além disso, também relataram uma tendência de melhora da qualidade de vida ao longo do tempo no grupo do TNT (p = 0,08) (Borg et al. 2020).

Outro estudo que fortalece a estratégia do TNT na prática atual é o RAPIDO trial que comparou a radioterapia de curta duração seguida pelo tratamento neoadjuvante com quimioterapia (CAPOX ou FOLFOX) por 4 meses com subsequente abordagem cirúrgica com o tratamento neoadjuvante com QRT convencional seguido de cirurgia e tratamento adjuvante opcional. O grupo submetido a radioterapia de curta duração mostrou duplicação da taxa de RPC (28% *versus* 14%; p<0,001) e aumento da sobrevida livre de metástases à distância em 7% em 3 anos (26,8% *versus* 20%; p= 0,005), com eventos adversos comparáveis e nenhum aumento de complicações na cirurgia ou pós-operatórias (Bahadoer et al. 2021).

Resultados preliminares da OPRA apresentados na ASCO 2020 demonstraram uma taxa significativamente maior de preservação de esfíncter no braço com TNT de consolidação (QRT seguido de quimioterapia) em comparação com o braço do TNT de indução (quimioterapia seguido de QRT) (59% *versus* 43%), mas nenhuma diferença significativa a respeito da SLD ou de metástases à distância em 3 anos (Garcia-Aguilar et al. 2020).

Sendo assim, TNT parece ser uma estratégia de tratamento promissora por aumentar as chances de resposta patológica e com isso, maiores taxas de preservação de órgão, além de proporcionar incremento na SLD. Porém, as vantagens do TNT também precisam ser equilibradas com a administração excessiva de terapia citotóxica e a longo prazo complicações tóxicas em pacientes com doença de baixo risco. Alguns pacientes com CRLA podem apresentar respostas muito boas e excelente controle local com QRT convencional.

Então, é fundamental que estudos futuros avaliem possíveis biomarcadores capazes de identificar pacientes com maior probabilidade de se beneficiar do TNT.

1.4.7 A importância da resposta patológica completa

Alguns pesquisadores procuraram determinar a relação entre regressão tumoral após tratamento neoadjuvante com os desfechos clínicos para assim justificar tratamentos mais agressivos no pré-operatório.

Amostras de 144 pacientes com câncer de reto cT3, cT4 que realizaram tratamento pré-operatório com QRT foram revisados retrospectivamente e determinado o grau de regressão tumoral (GRT) por análise patológica. O GRT foi então relacionado com fatores clínicos pré-tratamento e com os defechos de sobrevida apresentadas. Foi encontrado, por análise univariada, que o GRT é um fator preditor para falência local, sobrevida livre de metástase e SG (Vecchio et al. 2005).

Um outro estudo analisou amostras de 386 pacientes do estudo alemão CAO/ARO/AIO-94, submetidos a tratamento com QRT pré-operatória, e procurou relacionar o grau de regressão tumoral com os parâmetros clinico patológicos e os desfechos de sobrevida. Os autores relataram que aqueles pacientes que obtiveram regressão tumoral completa, ou seja, ausência de células tumorais viáveis, apresentaram SLD e incidência cumulativa de metástase à distância em 10 anos de 89,5% e 10,5%, respectivamente. Em contraponto, os pacientes que obtiveram uma regressão tumoral mínima apresentaram SLD e incidência cumulativa de metástase à distância em 10 anos de 63% e 39,6%, respectivamente. Na análise multivariada, os autores encontraram que linfonodo residual positivo e o GRT foram os únicos fatores prognósticos independentes para a incidência cumulativa de metástase à distancia e SLD. Já a recorrência local foi significantemente afetada pelo *status* patológico linfonodal e invasão linfática (Fokas et al. 2014).

Um estudo coreano investigou os resultados a longo prazo de pacientes com CRLA submetidos à QRT pré-operatória e que obtiveram RPC no tumor primário. Também foi analisado o significado prognóstico do *status* linfonodal patológico. Trezentos e sessenta e dois pacientes foram avaliados. Foi encontrada uma SG mediana em 5 anos de 92,8% e uma SLD em 5 anos de 84,6%. O status linfonodal foi ypT0N0 em 91,3%; ypT0N1 em 6,6% e ypT0N2 em 2,1%. A SG e SLD em 5 anos foram, respectivamente, 94,8% e 88,5% para os pacientes ypT0N0; e 72,8% e 45,2% para aqueles pacientes ypT0N+. Os autores encontraram como fator prognóstico mais relevante para a SG e SLD, nos pacientes com RPC no tumor primário, o *status* linfonodal (Yeo et al. 2010). Os dados corroboram com os estudos acima citados.

Com isso, uma estratégia racional para melhorar o prognóstico dos pacientes com CRLA, bem como reduzir a taxa de recorrência à distância, e assim aumentar a taxa de cura, seria a instituição de tratamento sistêmico mais intenso e de forma precoce que possibilitasse uma redução tumoral tanto na lesão primária como nos linfonodos de forma mais agressiva.

1.4.8 Tratamento Adjuvante

Embora na maioria dos estudos que avaliaram o papel do tratamento neoadjuvante com quimioterapia e radioterapia os pacientes tenham recebido quimioterapia adjuvante após a cirurgia definitiva, este ainda permanece um tópico de intenso debate na literatura.

O estudo europeu randomizado EORTC 22921 publicado em 2006, não demonstrou diferença de SG, independentemente da aplicação ou não de QT combinada ou adjuvante porém, observou-se tendência em favor da QT adjuvante na SLD em 5 anos em favor da adjuvância. Atualização desse estudo com seguimento mediano de 10,4 anos mostrou taxa de SG em 10 anos de 51,8% no grupo com quimioterapia adjuvante versus 48,4% no grupo sem quimioterapia (p=0,32) (Bosset et al. 2014).

A indicação da oxaliplatina em combinação 5-FU ou capecitabina adjuvante após tratamento trimodal foi avaliado em três estudos randomizados. No estudo CAO/ARO/AIO-04, a taxa de SLD em 3 anos foi superior no braço com adição de oxaliplatina adjuvante em comparação a apenas 5-FU (75,9 *versus* 71,2%, p=0,03) (Collette et al. 2007).

O estudo ADORE, de fase II, descreveu que a taxa de SLD em 3 anos foi superior no braço com oxaliplatina adjuvante (71,6 versus 62,9%, p=0,047). Na análise de subgrupo, pacientes com yp III (ypN1b, ypN2) e tumores que responderam pouco ao tratamento neoadjuvante se beneficiaram mais de FOLFOX do que 5-FU (Hong et al. 2014). Em sua

atualização recentemente publicada, a taxa de SLD em 6 anos foi de 68,2% no braço FOLFOX vs 56,8% no braço do 5-FU (p=0,018). A taxa de sobrevida geral de 6 anos foi de 78,1% no braço FOLFOX vs 76,4% no braço 5-FU (p = 0,21) (Hong et al. 2019).

Já o estudo PETACC-6, na análise interina planejada não demonstrou benefício com a adição de oxaliplatina a capecitabina no cenário adjuvante, com taxa de SLD em 3 anos de 73,9 versus 74,5%, p=0,78 (Schmoll et al. 2014).

Em metanálise incluindo dados de 1196 pacientes com estágio patológico II ou III submetidos a ressecção R0 não foi demonstrada diferença significativa na SG entre os pacientes que receberam quimioterapia adjuvante e aqueles que foram submetidos à seguimento (Breugom et al. 2015).

1.4.9 Estratégia de preservação de esfíncter

Para vários outros tipos de tumor, incluindo carcinoma anal, laríngeo, cervical e pulmonar, dependendo do estágio e de uma variedade de fatores clínicos o tratamento com QRT é utilizado como estratégia definitiva sem complementação com cirurgia. No CRLA, a obtenção da resposta clinica completa (RCC) levanta a questão de saber se a cirurgia pode ser evitada (Maas et al. 2011). O principal motivo para evitar a abordagem cirúrgica são as morbidades pós-operatórias, incluindo incontinência urinária, disfunção sexual e fecal de longa duração, além da necessidade de estomas temporários ou permanentes associados ao procedimento. A mortalidade pós-operatória também pode ser substancial, dependendo das comorbidades médicas e da idade do paciente (Smith et al. 2015).

A estratégia conhecida como “watch and wait” (WW) foi introduzida por Habr-Gama et al., que relataram uma SG altamente promissora de 97,7% e SLD de 84% após uma década de acompanhamento naqueles que não foram submetidos a cirurgia após atingirem RCC (Habr-Gama et al. 2004). Sendo assim, o papel da cirurgia após o tratamento neoadjuvante para os pacientes com RCC passou a ser questionado, com o objetivo de evitar sequelas do tratamento cirúrgico radical.

Estudo multicêntrico internacional também relatou resultados favoráveis para aqueles com RCC que optam pela estratégia de WW, com uma taxa de sobrevivência específica da doença de 94% com apenas 8% dos pacientes que desenvolveram metástases à distância em 5 anos (van der Valk et al. 2018).

Martens et al. (2016) relataram resultados de 100 pacientes que não foram

submetidos a cirurgia após atingirem uma RCC com tratamento neoadjuvante. Destes 100 pacientes, 15 apresentaram recorrência local após um acompanhamento médio de mais de 3 anos. Doze dessas 15 recorrências locais foram isoladas, sem metástases à distância, e foram passíveis de cirurgia de resgate (Martens et al. 2016).

Por outro lado, meta-análise com onze estudos que incluíram 1.131 pacientes, dos quais 412 pacientes foram submetidos à estratégia WW, 678 pacientes foram submetidos a cirurgia e 41 pacientes foram submetidos a ressecção local. O grupo WW teve uma taxa maior de recorrência local do que o grupo da cirurgia (OR 7,32, IC 95% 3,58 a 14,95, p <0,001). No entanto, o grupo WW apresentou SLD e SG semelhantes em comparação com o grupo da cirurgia e o grupo da ressecção local (Zhao et al. 2020).

O principal impedimento para considerar a omissão de cirurgia deve-se a preocupação com a ausência de dados randomizados além das limitações para avaliação precisa da regressão tumoral com exames de imagem e anatomopatológico. Com isso, ao considerar a estratégia de preservação de órgãos, uma questão importante é identificar os pacientes que apresentam regressão tumoral significativa com tratamento neoadjuvante e esta abordagem é bastante complexa e desafiadora, sendo necessária a incorporação de novas ferramentas que permitam a melhor identificação destes pacientes.

1.5 CÉLULAS TUMORAIS CIRCULANTES

A disseminação do câncer necessita de Células Tumorais Circulantes (CTCs) (Park et al. 2011), que são células raras circundadas por inúmeras células hematopoiéticas presentes na circulação sanguínea (San Juan et al. 2019). As CTCs são extremamente escassas em comparação com as células sanguíneas normais, com apenas 1–100 CTCs presentes entre milhões de leucócitos e bilhões de eritrócitos (Mego et al. 2010; Low et al. 2015; van der Toom et al. 2016).

A presença de CTCs no sangue periférico foi relatada pela primeira vez por Ashworth (1869), um médico austríaco, quando fazia a necrópsia de um paciente com tumores subcutâneos metastáticos, situados na parede anterior do tórax e do abdômen. Ele observou células na circulação idênticas às provenientes dos tumores e postulou que se estas células eram oriundas de uma estrutura tumoral existente.

Embora CTCs carreguem informações robustas sobre o tumor e tenham sido estudadas extensivamente nos últimos anos, sua biologia ainda carece de novos estudos (Mamdouhi et al. 2019). A presença de CTCs no sangue pode ser considerada como um potencial fator prognóstico além de predizer resistência a estratégias terapêuticas (Rahbari et al. 2010). Quantidades elevadas de CTCs têm sido relatadas na literatura como fator de prognóstico ruim (piores SLP e SG) em alguns tumores metastáticos como mama, colorretal e próstata (Cristofanilli et al. 2004; De Bono et al. 2008; Cohen et al. 2009; Tol et al. 2010). Além disso, as CTCs podem também fornecer dados preditivos, como os fornecidos por meio da análise da expressão de marcadores de resistência a tratamento (Stoecklein et al. 2008; Gazzaniga et al. 2010; Gradilone et al. 2011; Shi et al. 2020), e com isso ser um dos fatores determinantes de recorrência local e progressão tumoral. Sendo assim, a detecção de CTCs pode nos ajudar a identificar pacientes com necessidade de terapias sistêmicas adicionais antes ou após um tratamento definitivo local, como ressecção cirúrgica ou radioterapia do tumor primário. Desta forma, incluir a detecção ou contagem de CTCs na análise dos pacientes tornaria o tratamento mais personalizado (Abdallah et al. 2021).

Com relação ao CCR, sabe-se que a primeira estratégia para o tratamento desta doença é a ressecção completa da lesão, o que algumas vezes inclui ressecção dos órgãos circunvizinhos e ampla dissecção linfonodal, procedimentos que afetam a qualidade de vida dos pacientes (Katsumata et al. 2006). Contudo, apesar da ressecção radical, alguns pacientes apresentam recorrência, que se acredita ser devido a sítios de micrometástases residuais (Haraldsdottir et al. 2014).

Estudo realizado por Cohen et al. (2009), incluiu 430 pacientes com CCRm entre 2004 e 2006. As CTCs foram enumeradas por separação magnética (CellSearch^{TM} System, Veridex LLC, Raritan, NJ) e os autores classificaram os pacientes de acordo com os níveis de CTCs, utilizando como *cut-off* o valor de 3 CTCs/7,5 mL de sangue. Avaliando SLP e SG eles observaram que pacientes com CTCs desfavoráveis (≥3 CTCs) tiveram menores sobrevidas ($p= 0.004$, para SLP e $p<0.0001$, para SG), mostrando a possibilidade de monitoramento dos pacientes de acordo com os níveis de CTCs encontrados.

Sastre et al. (2008) observaram uma correlação positiva entre o número de CTCs e o estádio clínico em 97 pacientes com as seguintes características: CCR não metastático recém diagnosticado ou com câncer de reto sem quimioterapia ou radioterapia neoadjuvantes; CCRm recém diagnosticado; CCR recidivado no seguimento. Usou como grupo controle 30

pacientes saudáveis. Como *cut-off* foi escolhido o valor de 2 CTCs/7,5 mL para definir um teste como positivo. Não foi observada qualquer relação entre CTCs e localização do tumor primário, níveis aumentados de CEA, de lactato desidrogenase ou grau de diferenciação.

Utilizando também 2 CTCs/7,5 mL como *cut-off*, Romiti et al. (2014) realizaram um estudo com 75 pacientes com câncer colorretal, sendo 72% estádios I-III e 28% estádio IV. O grupo isolou CTCs usando um método imunomagnético e observaram pior SG no grupo de pacientes com CTCs acima de 2/7,5 mL (36,2 meses *versus* 61,6 meses; p= 0,002). Ainda, observaram que os pacientes que mantinham CTCs positivas mesmo após o tratamento tinham pior prognóstico, e as CTCs foram fator prognóstico independente de SG (p= 0,03; HR: 3,5).

Em um estudo prospectivo com 54 pacientes com CCRm, foi avaliado o impacto da cinética CTCs durante o tratamento. CTC 1 (*baseline*) *versus* CTC realizada dois meses após o início do tratamento (CTC2). Pacientes com CTC1 positiva (CTCs acima da mediana) e que se tornaram negativos (CTCs abaixo da mediana), considerados como cinética favorável, tiveram melhor SLP, comparada com os pacientes que na CTC1 eram negativos (CTCs abaixo da mediana) e que durante o tratamento apresentaram CTC 2 positiva (CTCs acima da mediana) e foram considerados como cinética desfavorável (p=0.06). Além disso, os pacientes cujos estudos de imagem mostraram progressão radiológica tiveram uma quantificação aumentada de CTCs na segunda coleta (CTC 2) em comparação com aqueles sem progressão (p = 0,04) (Souza e Silva et al. 2016).

Em recente publicação com 75 pacientes com CCRm a avaliação da cinética das CTCs se mostrou promissora. Uma diferença de CTC2-CTC1> 5,5 CTCs / mL foi associada a uma SLP mediana reduzida. Na análise multivariada, CTC1> 1,5 CTCs/mL foi um fator prognóstico independente para pior SG (Silva et al. 2021).

Diante da evolução de métodos mais eficientes e sensíveis para detecção de CTCs, criou-se a possibilidade de identificação destas células em pacientes não metastáticos, o que favorece sua aplicabilidade em estudos de neoadjuvância e adjuvância (Chen et al. 2017; Tsai Ws et al. 2016).

Para o câncer de reto ainda temos carência de estudos que avaliam a aplicabilidade da CTCs (Magni et al. 2014). Sun et al. (2016) demonstraram em estudo com 115 pacientes com diagnóstico de CRLA (cT3-4 e/ou N+) que a detecção de CTCs é uma ferramenta útil para avaliar e prever respostas ao tratamento neoadjuvante.

Um estudo prospectivo com 267 pacientes com CRLA tratados com QRT, avaliou o impacto de CTCs positivas como um possível marcador de resposta ao tratamento neoadjuvante. A regressão histopatológica após QRT foi evidente em 27,8% dos pacientes. No subgrupo de respondedores após o tratamento neoadjuvante, foram encontradas CTCs positivas em uma taxa significativamente menor do que nos não respondedores (p = 0,03). Nenhuma associação significativa foi demonstrada entre a detecção de CTC e as características do tumor. Os respondedores após QRT neoadjuvante tiveram uma incidência menor de CTC em comparação com aqueles que não responderam, o que pode ser o resultado de um tratamento sistêmico e local eficaz antes da cirurgia (Hinz et al. 2015). Em particular, na avaliação *baseline,* os pacientes que responderam ao tratamento neoadjuvante tiveram uma taxa de detecção de CTC significativamente maior em comparação com os que não responderam. No entanto, a taxa de detecção de CTC diminuiu significativamente após a QRT para os respondedores.

1.6 MARCADORES DE RESISTÊNCIA AO TRATAMENTO

1.6.1 RAD23B

A Radiação Ionizante (RI) é um importante pilar do tratamento neoadjuvante com QRT para pacientes com CRLA, com o objetivo de proporcionar dano e morte celular. No entanto, nem todas as células tumorais apresentam radiossensibilidade, uma vez que existem mecanismos capazes de reparar efetivamente seu DNA danificado (Hubenak et al. 2014; Shen et al. 2018). Portanto, a compreensão desses mecanismos é fundamental para a busca de possíveis biomarcadores de resistência a essa estratégia terapêutica.

O homólogo B de RAD23 (RAD23B) é uma proteína de reparo do DNA, motivo pelo qual alguns estudos correlacionam sua expressão com resistência à RI (Dantuma et al. 2009). Em estudo em câncer gástrico, Shen et al. (2018) demonstraram que ao proporcionar a inibição do RAD23B estaríamos aumentando o efeito do tratamento da radiação nas células cancerosas dos tumores gástricos, tornando estas mais sensíveis a este tratamento.

A expressão de RAD23B está elevada em alguns tumores de cabeça e pescoço como o de laringe (Abbasi et al. 2009), mas no câncer retal, até o momento, não há estudos que avaliem esse possível marcador para predição de resposta.

1.6.2 TYMS

A timidilato sintase (TYMS) é uma enzima que participa de uma das vias de metabolismo do 5-FU. A quantidade da expressão da TYMS no tumor parece ser um preditor de resposta dos pacientes tratados com 5-FU. Alguns estudos descrevem que pacientes com CCRm com altos níveis de TYMS não respondem ao tratamento infusional com 5-FU, enquanto aqueles com baixos níveis apresentam melhores taxas de resposta (Kim et al. 2009; Calascibetta et al. 2010).

Contudo, apesar de muitos estudos relatarem SG ruim em tumores com alta expressão de TYMS, o real valor prognóstico desta enzima ainda não está estabelecido e validado para sua aplicabilidade clínica (Allegra et al. 2002; Chen et al. 2012).

Estudo com 54 pacientes com CCRm tratados com 5-FU demonstrou que a expressão da TYMS nas CTCs foi positiva em 9 (26,5%). Seis desses pacientes apresentaram progressão do tumor após o tratamento com 5-FU (p= 0,07). Estes resultados sugerem que a análise de TYMS pode ser uma ferramenta útil como um biomarcador preditor de resistência a 5-FU se analisadas em CTCs de pacientes com CCRm (Abdallah et al. 2015).

No câncer retal, estudos anteriores demonstraram que a baixa expressão do gene TYMS correlacionou-se com a resposta patológica ao tratamento neoadjuvante com QRT à base de 5-FU, porém existem dados conflitantes que merecem estudos que avaliem a TYMS como possível biomarcador preditor de resposta ao tratamento neoadjuvante (Okonkwo et al. 2001; Saw et al. 2003; Jakob et al. 2008).

1.6.3 Fator Transformador de Crescimento-Beta

Algumas moléculas podem servir como guia para traçar o perfil de agressividade tumoral e metastatização, como a expressão do receptor do *Tumor Growth Factor β* (TGF-βR) nas células tumorais. O TGF-β está envolvido no processo de transição epitélio-mesênquima, pela ativação de vias supressoras de E-caderina, como SNAIL e SLUG (Massagué 2008).

A expressão de TGF-β e mutações nos genes codificadores do seu receptor podem ter correlação com a atividade oncogênica desta citocina, assim como com aumento de progressão tumoral e alto potencial de migração celular (Zarzynska 2014). O TGF- β derivado de plaquetas é um elemento essencial para o evento metastático, especificamente na transição epitélio-mesênquima, que permite a disseminação para locais distantes. As alterações morfológicas deste processo são transitórias e reversíveis, ocorrem para promover

motilidade celular e disseminação do tumor para fora de seu ambiente (Thiery 2002; Goulet et al. 2021). Em ratos com deficiência de plaquetas e de TGF-β, o extravasamento de células de tumor e metástases foi diminuído, indicando que a libertação de TGF-β aumenta a atividade de invasão e metástase (Labelle et al. 2011).

O TGF-β pode atuar de diversas maneiras, favorecendo metástase tumoral, com a migração, angiogênese e invasão do tumor. É demonstrado que a sua superprodução precede a formação do tumor e prepara um microambiente favorável para células cancerosas (Ellenrieder et al. 2001; Gasparini-Junior et al. 2019).

1.7 RESPOSTA IMUNOLÓGICA E CÂNCER

A principal razão da mortalidade relacionada ao câncer é a disseminação de células cancerosas para locais distantes. Contudo, apenas alguns pequenos grupos de células tumorais podem formar metástases, com sucesso, por adquirirem mecanismos para diminuir a resposta imune, escapando assim da vigilância imunológica (Zhang et al. 2016). Esta condição imunossupressora contribui para a metástase em múltiplos passos, tais como migração de células tumorais, sobrevivência das CTCs e crescimento das células tumorais metastáticas. Embora as células tumorais possam entrar na circulação nas primeiras fases do desenvolvimento do câncer, a detecção de metástases só ocorre em estádios avançados (Nemeth et al. 2003).

Células do sistema imune são capazes de eliminar diretamente as CTCs, contudo, evidências crescentes vêm demonstrando que em ambos os modelos (animais e humanos) o tumor primário pode alterar a mielopoiese, convertendo células mielóides em células imunossupressoras potentes, entre elas os neutrófilos patologicamente ativados (neutrófilos associados ao tumor-NATs) (Ostrand-Rosenberg 2008; Gabrilovich et al. 2012). NATs têm sido relatados como produtores de fatores angiogênicos e de enzimas que degradam matriz extracelular, o que suporta o fenótipo de metástases e suprime a resposta imune anti-tumor. A maioria dos estudos concentrou-se sobre as funções de NATs no microambiente tumoral. No entanto, está cada vez mais claro que NATs atuam além deste microambiente, desempenhando múltiplos passos durante a progressão tumoral (Demers et al. 2013).

Heo et al. (2016), demostraram em estudo prospectivo com 10 pacientes com CRLA submetidos a tratamento neoadjuvante que a modificação entre as contagens de

subpopulações de linfócitos circulantes durante o tratamento pode determinar correlação preditiva a resposta ao tratamento proposto. Ou seja, em pacientes com redução da contagem da população de células "natural killer" durante o tratamento com a radioterapia observou-se correlação com a resposta patológica linfonodal (p=0,034).

Em relação a terapias imunológicas, inicialmente o Pembrolizumabe e Nivolumabe foram aprovados como drogas inibidoras da família dos imuno *check-points* PD1/PDL1 pelo FDA, para melanoma refratário a Ipilimumabe (Brahmer et al. 2015; Vaddepally et al. 2020). Resumidamente, PD-1 (do inglês, *Programmed Death*-1) é receptor expresso por células T ativadas, considerado um ponto chave de controle da resposta imune, que medeia sua imunossupressão. PD-1 age principalmente nos tecidos periféricos, em que as células T podem encontrar seus imunossupressores (células tumorais, células do estroma ou ambas) que contêm PD-1 ligantes (PD-L1/ B7-H1 e PD-L2/B7-DC). A inibição da interação entre PD-1e PD-L1 pode aumentar as respostas de células T *in vitro* (Iwai et al. 2002; Topalian et al. 2012; Zam et al. 2021).

O uso de inibidores de PD-1 e PD-L1 em outros tipos de tumores (pulmão, mama, bexiga e rins, por exemplo) tem produzido bons resultados em termos de toxicidade e atividade anti-tumoral (Philips et al. 2015; Shin et al. 2015; Hegde et al. 2020). A expressão de PD-L1 pelas células tumorais, por IHQ, após o bloqueio de PD-1, tem sido avaliada como potencial biomarcador de resposta terapêutica, mas tem mostrado limitações técnicas. Além disto, há uma correlação fraca entre marcações IHQ de tumores primários e metástases, mostrando que o tumor primário não é um substituto adequado para verificar expressão de PD-L1 em sítios metastáticos, e assim, definir tratamento (Jilaveanu et al. 2014; Yoshimura et al. 2019; Rozenblit et al. 2020).

Mazel et al. (2015), em estudo realizado com 16 pacientes com câncer de mama, isolando CTCs pelo *CellSearch System*, encontraram expressão de PD-L1 por imunocitoquimica em CTCs de 11 pacientes (68,8%). A intenção do grupo agora é verificar se esta expressão se correlaciona com resposta ao tratamento e usá-la como "biópsia líquida".

A expressão do PD-L1 no câncer retal ainda é pouco estudada, mas alguns autores têm demonstrado que a radioterapia pode estimular sua expressão, embora seu impacto prognóstico e preditivo ainda seja desconhecido (Saigusa et al. 2016; Chiang et al. 2019; Chen et al. 2019; Boustani et al. 2020; Huemer et al. 2020).

2 OBJETIVOS

2.1 OBJETIVO PRIMÁRIO

Analise da expressão por Imunocitoquímica de RAD23B e TYMS nas CTCs do sangue periférico de pacientes com câncer de reto localmente avançado (cT3/T4 e ou cN+) submetidos a tratamento neoadjuvante com quimiorradioterapia e correlacionar sua ausência de expressão com a resposta patológica completa e, assim, identificar possíveis respondedores ao tratamento.

2.2 OBJETIVOS SECUNDÁRIOS

- Verificar a influência da cinética das CTCs na resposta ao tratamento e correlacionar com a resposta patológica completa após a quimiorradioterapia neoadjuvante e com a sobrevida livre de progressão e sobrevida global para os pacientes com câncer de reto localmente avançado;
- Analisar nas CTCs marcadores de resposta imune (PD-L1) e de invasão (TGF-β) e correlacionar com evolução terapêutica e prognóstica.

3 ARTIGO SUBMETIDO

Artigo submetido na revista Cells em 30/04/2021 e publicado no dia 18/06/2021
Fator de impacto: 6.600

Article: Molecular and dynamic evaluation of proteins related to resistance to neoadjuvant treatment with chemoradiotherapy in circulating tumor cells of patients with locally advanced rectal cancer.

Link para acesso: https://www.mdpi.com/2073-4409/10/6/1539

4 CONCLUSÕES

- Com nosso estudo demostramos importante correlação entre a expressão do RAD23B nas CTCs (*baseline* e pós QRT) com a ausência de resposta ao tratamento neoadjuvante com QRT.
- Avaliamos que a expressão da TYMS nas CTCs também pode contribuir para avaliação preditiva de resposta ao tratamento neoadjuvante dos pacientes submetidos a QRT;
- Conseguimos demonstrar no cenário de doença localizada, conforme já havíamos demonstrado na doença metastática em estudos prévios do nosso grupo, a relevância prognóstica da cinética da CTCs. Este achado enfatiza a importância da avaliação dinâmica com as CTCs na estratificação prognóstica destes pacientes;
- Além da expressão do RAD23B e TYMS, conseguimos sugerir que a avaliação de outras proteínas na CTCs como TGF-βRI e PDL-1 podem contribuir na avaliação prognóstica e terapêutica destes pacientes;
- Reconhecemos as limitações do nosso estudo, como o tamanho da amostra, tempo de seguimento e a doença localizada. Pode ser que o fato de termos trabalhado com doença localizada tenha dificultado o isolamento das CTCs, impedindo a avaliação molecular durante o estudo de todos as proteínas sugeridas nos objetivos;
- Apesar destas limitações, nosso trabalho possibilitou o conhecimento de possíveis marcadores que devem ser avaliados em futuros estudos clínicos, a fim de aprimorar a avaliação de resposta do tratamento neoadjuvante, o manejo dos pacientes e assim individualizar o tratamento.

5 REFERÊNCIAS

Abbasi R, Ramroth H, Becher H, Dietz A, Schmezer P, Popanda O. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in ERCC5 , ERCC6 and RAD23B but not by polymorphisms in five other nucleotide excision repair genes. Int J Cancer. 2009 Sep 15; 125(6):1431–9.

Abdallah EA, Fanelli MF, Buim MEC, Machado Netto MC, Gasparini Junior JL, Souza e Silva V, et al. Thymidylate synthase expression in circulating tumor cells: A new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. Int J Cancer. 2015 Sep 11; 137(6):1397–405.

Abdallah EA, Fanelli MF, Souza e Silva V, Machado Netto MC, Gasparini Junior JL, Araújo DV, et al. MRP1 expression in CTCs confers resistance to irinotecan-based chemotherapy in metastatic colorectal cancer. Int J Cancer. 2016 Aug 15; 139(4):890–8.

Abdallah EA, Souza e Silva V, Braun AC, Gasparini VA, Kupper BEC, Tariki MS, et al. A higher platelet-to-lymphocyte ratio is prevalent in the presence of circulating tumor microemboli and is a potential prognostic factor for non-metastatic colon cancer. Transl Oncol. 2021 Jan; 14(1):100932.

Allegra CJ, Parr AL, Wold LE, Mahoney MR, Sargent DJ, Johnston P, et al. Investigation of the Prognostic and Predictive Value of Thymidylate Synthase, p53, and Ki-67 in Patients With Locally Advanced Colon Cancer. J Clin Oncol. 2002 Apr 1; 20(7):1735–43.

Araghi M, Soerjomataram I, Jenkins M, Brierley J, Morris E, Bray F, et al. Global trends in colorectal cancer mortality: projections to the year 2035. Int J Cancer. 2019 Jun 15; 144(12):2992–3000.

Attenberger UI, Winter J, Harder FN, Burkholder I, Dinter D, Kaltschmidt S, et al. Height of Rectal Cancer: A Comparison between Rectoscopic and Different MRI Measurements. Gastroenterol Res Pract. 2020 Jan 28; 2020:1–7.

Bahadoer RR, Dijkstra EA, van Etten B, Marijnen CAM, Putter H, Kranenbarg EM-K, et al. Short-course radiotherapy followed by chemotherapy before total mesorectal excision (TME) versus preoperative chemoradiotherapy, TME, and optional adjuvant chemotherapy in locally

advanced rectal cancer (RAPIDO): a randomised, open-label, phase 3 trial. Lancet Oncol. 2021 Jan; 22(1):29–42.

Beets-Tan RGH, Beets GL. Rectal Cancer: Review with Emphasis on MR Imaging. Radiology. 2004 Aug; 232(2):335–46.

Binefa G. Colorectal cancer: From prevention to personalized medicine. World J Gastroenterol. 2014; 20(22):6786.

de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating Tumor Cells Predict Survival Benefit from Treatment in Metastatic Castration-Resistant Prostate Cancer. Clin Cancer Res. 2008 Oct 1; 14(19):6302–9.

Borg C, Rullier E, Marchal F, Etienne P-L, Rio E, Francois E, et al. LBA21 Neoadjuvant mFOLFIRINOX and preoperative chemoradiation (CRT) versus preoperative CRT in patients with T3-4 rectal cancer: Surgical and quality of life results of PRODIGE 23 phase III trial. Ann Oncol. 2020 Sep; 31:S1152.

Bosset J-F, Calais G, Mineur L, Maingon P, Stojanovic-Rundic S, Bensadoun R-J, et al. Fluorouracil-based adjuvant chemotherapy after preoperative chemoradiotherapy in rectal cancer: long-term results of the EORTC 22921 randomised study. Lancet Oncol. 2014 Feb; 15(2):184–90.

Boustani J, Derangère V, Bertaut A, Adotevi O, Morgand V, Charon-Barra C, et al. Radiotherapy Scheme Effect on PD-L1 Expression for Locally Advanced Rectal Cancer. Cells. 2020 Sep 10; 9(9):2071.

Brahmer JR, Hammers H, Lipson EJ. Nivolumab: targeting PD-1 to bolster antitumor immunity. Futur Oncol. 2015 May; 11(9):1307–26.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov; 68(6):394–424.

Breugom AJ, Swets M, Bosset J-F, Collette L, Sainato A, Cionini L, et al. Adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with rectal cancer: a systematic review and meta-analysis of individual patient data. Lancet Oncol. 2015 Feb; 16(2):200–7.

Bujko K, Nowacki MP, Nasierowska-Guttmejer A, Michalski W, Bebenek M, Kryj M. Long-term results of a randomized trial comparing preoperative short-course radiotherapy with

preoperative conventionally fractionated chemoradiation for rectal cancer. Br J Surg. 2006 Sep 19; 93(10):1215–23.

Calascibetta A, Contino F, Feo S, Gulotta G, Cajozzo M, Antona A, et al. Analysis of the Thymidylate Synthase Gene Structure in Colorectal Cancer Patients and Its Possible Relation with the 5-Fluorouracil Drug Response. J Nucleic Acids. 2010; 2010:1–4.

Campelo P, Barbosa E. Functional outcome and quality of life following treatment for rectal cancer. J Coloproctology. 2016 Dec 17; 36(04):251–61.

Cercek A, Roxburgh CSD, Strombom P, Smith JJ, Temple LKF, Nash GM, et al. Adoption of Total Neoadjuvant Therapy for Locally Advanced Rectal Cancer. JAMA Oncol. 2018 Jun 14; 4(6):e180071.

Chen F, Wang S, Fang Y, Zheng L, Zhi X, Cheng B, et al. Feasibility of a novel one-stop ISET device to capture CTCs and its clinical application. Oncotarget. 2017 Jan 10; 8(2):3029–41.

Chen T-W, Huang KC-Y, Chiang S-F, Chen WT-L, Ke T-W, Chao KSC. Prognostic relevance of programmed cell death-ligand 1 expression and CD8+ TILs in rectal cancer patients before and after neoadjuvant chemoradiotherapy. J Cancer Res Clin Oncol. 2019 Apr 14; 145(4):1043–53.

Chen Y, Yi C, Liu L, Li B, Wang Y, Wang X. Thymidylate Synthase Expression and Prognosis in Colorectal Cancer: A Meta-Analysis of Colorectal Cancer Survival Data. Int J Biol Markers. 2012 Jul 15; 27(3):203–11.

Chiang S-F, Huang C-Y, Ke T-W, Chen T-W, Lan Y-C, You Y-S, et al. Upregulation of tumor PD-L1 by neoadjuvant chemoradiotherapy (neoCRT) confers improved survival in patients with lymph node metastasis of locally advanced rectal cancers. Cancer Immunol Immunother. 2019 Feb 17; 68(2):283–96.

Cohen SJ, Punt CJA, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. Ann Oncol. 2009 Jul; 20(7):1223–9.

Collette L, Bosset J-F, den Dulk M, Nguyen F, Mineur L, Maingon P, et al. Patients With Curative Resection of cT3-4 Rectal Cancer After Preoperative Radiotherapy or Radiochemotherapy: Does Anybody Benefit From Adjuvant Fluorouracil-Based Chemotherapy? A Trial of the European Organisation for Research and Treatment of Cancer Rad. J Clin Oncol. 2007 Oct 1; 25(28):4379–86.

Conroy T, Bosset J-F, Etienne P-L, Rio E, François É, Mesgouez-Nebout N, et al. Neoadjuvant chemotherapy with FOLFIRINOX and preoperative chemoradiotherapy for patients with

locally advanced rectal cancer (UNICANCER-PRODIGE 23): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol. 2021 May; 22(5):702–15.

Cotterchio M, Manno M, Klar N, McLaughlin J, Gallinger S. Colorectal Screening is Associated with Reduced Colorectal Cancer Risk: A Case–Control Study within the Population-Based Ontario Familial Colorectal Cancer Registry. Cancer Causes Control. 2005 Sep; 16(7):865–75.

Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. N Engl J Med. 2004 Aug 19; 351(8):781–91.

Curvo-Semedo L. Rectal Cancer. Magn Reson Imaging Clin N Am. 2020 Feb; 28(1):105–15.

Dantuma NP, Heinen C, Hoogstraten D. The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. DNA Repair (Amst). 2009 Apr 5; 8(4):449–60.

Delibegovic S. Introduction to Total Mesorectal Excision. Med Arch. 2017; 71(6):434.

Demers M, Wagner DD. Neutrophil extracellular traps. Oncoimmunology. 2013 Feb 28; 2(2):e22946.

Deng Y, Chi P, Lan P, Wang L, Chen W, Cui L, et al. Neoadjuvant Modified FOLFOX6 With or Without Radiation Versus Fluorouracil Plus Radiation for Locally Advanced Rectal Cancer: Final Results of the Chinese FOWARC Trial. J Clin Oncol. 2019 Dec 1; 37(34):3223–33.

Deng Y, Chi P, Lan P, Wang L, Cui L, Chen D, et al. A multi-center randomized controlled trial of mFOLFOX6 with or without radiation in neoadjuvant treatment of local advanced rectal cancer (FOWARC study): Preliminary results. J Clin Oncol. 2015 May 20; 33(15_suppl):3500–3500.

Doubeni CA, Laiyemo AO, Major JM, Schootman M, Lian M, Park Y, et al. Socioeconomic status and the risk of colorectal cancer. Cancer. 2012 Jul 15; 118(14):3636–44.

Ellenrieder V, Hendler SF, Boeck W, Seufferlein T, Menke A, Ruhland C, et al. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res. 2001 May 15; 61(10):4222–8.

Engelen SME, Maas M, Lahaye MJ, Leijtens JWA, van Berlo CLH, Jansen RLH, et al. Modern multidisciplinary treatment of rectal cancer based on staging with magnetic resonance imaging leads to excellent local control, but distant control remains a challenge. Eur J Cancer. 2013 Jul; 49(10):2311–20.

Erlandsson J, Holm T, Pettersson D, Berglund Å, Cedermark B, Radu C, et al. Optimal fractionation of preoperative radiotherapy and timing to surgery for rectal cancer (Stockholm III): a multicentre, randomised, non-blinded, phase 3, non-inferiority trial. Lancet Oncol. 2017 Mar; 18(3):336–46.

Fang M, Marta GN. Hypofractionated and hyper-hypofractionated radiation therapy in postoperative breast cancer treatment. Rev Assoc Med Bras. 2020 Sep; 66(9):1301–6.

Feeney G, Sehgal R, Sheehan M, Hogan A, Regan M, Joyce M, et al. Neoadjuvant radiotherapy for rectal cancer management. World J Gastroenterol. 2019 Sep 7; 25(33):4850–69.

Fernandez-Martos C, Garcia-Albeniz X, Pericay C, Maurel J, Aparicio J, Montagut C, et al. Chemoradiation, surgery and adjuvant chemotherapy versus induction chemotherapy followed by chemoradiation and surgery: long-term results of the Spanish GCR-3 phase II randomized trial. Ann Oncol. 2015 Aug; 26(8):1722–8.

Fokas E, Liersch T, Fietkau R, Hohenberger W, Beissbarth T, Hess C, et al. Tumor Regression Grading After Preoperative Chemoradiotherapy for Locally Advanced Rectal Carcinoma Revisited: Updated Results of the CAO/ARO/AIO-94 Trial. J Clin Oncol. 2014 May 20; 32(15):1554–62.

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012 Apr 22; 12(4):253–68.

Garcia-Aguilar J, Patil S, Kim JK, Yuval JB, Thompson H, Verheij F, et al. Preliminary results of the organ preservation of rectal adenocarcinoma (OPRA) trial. J Clin Oncol. 2020 May 20; 38(15_suppl):4008–4008.

Gasparini-Junior JL, Fanelli MF, Abdallah EA, Chinen LTD. Evaluating MMP-2 and TGFß-RI expression in circulating tumor cells of pancreatic cancer patients and their correlation with clinical evolution. ABCD Arq Bras Cir Dig (São Paulo). 2019; 32(2).

Gazzaniga P, Naso G, Gradilone A, Cortesi E, Gandini O, Gianni W, et al. Chemosensitivity profile assay of circulating cancer cells: prognostic and predictive value in epithelial tumors. Int J Cancer. 2010; 126(10):2437-47.

Gérard J-P, Azria D, Gourgou-Bourgade S, Martel-Lafay I, Hennequin C, Etienne P-L, et al. Clinical Outcome of the ACCORD 12/0405 PRODIGE 2 Randomized Trial in Rectal Cancer. J Clin Oncol. 2012 Dec 20; 30(36):4558–65.

Glimelius B, Tiret E, Cervantes A, Arnold D. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2013 Oct; 24:vi81–8.

Goulet CR, Pouliot F. TGFβ Signaling in the Tumor Microenvironment. Adv Exp Med Biol. 2021; 1270:89-105.

Gradilone A, Naso G, Raimondi C, Cortesi E, Gandini O, Vincenzi B, et al. Circulating tumor cells (CTCs) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization. Ann Oncol. 2011 Jan; 22(1):86–92.

Habr-Gama A. Câncer coloretal: a importância de sua prevenção. Arq Gastroenterol. 2005 Mar; 42(1):2–3.

Habr-Gama A, Perez RO, Nadalin W, Sabbaga J, Ribeiro U, Silva e Sousa AH, et al. Operative Versus Nonoperative Treatment for Stage 0 Distal Rectal Cancer Following Chemoradiation Therapy. Ann Surg. 2004 Oct; 240(4):711–8.

Haraldsdottir S, Einarsdottir HM, Smaradottir A, Gunnlaugsson A, Halfdanarson TR. Colorectal cancer - review. Laeknabladid. 2014; 100(2):75-82.

Heald R., Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. Lancet. 1986 Jun; 327(8496):1479–82.

Hegde PS, Chen DS. Top 10 Challenges in Cancer Immunotherapy. Immunity. 2020 Jan; 52(1):17–35.

Heo J, Oh Y-T, Noh OK, Chun M, Park J-E, Cho S-R. Nodal tumor response according to the count of peripheral blood lymphocyte subpopulations during preoperative chemoradiotherapy in locally advanced rectal cancer. Radiat Oncol J. 2016 Dec 31; 34(4):305–12.

Hinz S, Röder C, Tepel J, Hendricks A, Schafmayer C, Becker T, et al. Cytokeratin 20 positive circulating tumor cells are a marker for response after neoadjuvant chemoradiation but not for prognosis in patients with rectal cancer. BMC Cancer. 2015 Dec 16; 15(1):953.

Hong YS, Kim SY, Lee JS, Nam B-H, Kim K, Kim JE, et al. Oxaliplatin-Based Adjuvant Chemotherapy for Rectal Cancer After Preoperative Chemoradiotherapy (ADORE): Long-Term Results of a Randomized Controlled Trial. J Clin Oncol. 2019 Nov 20; 37(33):3111–23.

Hubenak JR, Zhang Q, Branch CD, Kronowitz SJ. Mechanisms of Injury to Normal Tissue after Radiotherapy. Plast Reconstr Surg. 2014 Jan; 133(1):49e-56e.

Huemer F, Klieser E, Neureiter D, Schlintl V, Rinnerthaler G, Pagès F, et al. Impact of PD-L1 Scores and Changes on Clinical Outcome in Rectal Cancer Patients Undergoing Neoadjuvant Chemoradiotherapy. J Clin Med. 2020 Aug 27; 9(9).

INCA. Ministério da Saúde. Instituto Nacional de Câncer José Alencar Gomes da Silva. Estimativa /2020: Incidência de câncer no Brasil [Internet]. Rio de Janeiro; 2019. Available from: https://www.inca.gov.br/sites/ufu.sti.inca.local/files/media/document/estimativa-2020-incidencia-de-cancer-no-brasil.pdf

Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci. 2002 Sep 17; 99(19):12293–7.

Jakob C, Liersch T, Meyer W, Becker H, Baretton GB, Aust DE. Neuro-regulation of lower esophageal sphincter Predictive value of Ki67 and p53 in locally advanced rectal cancer: Correlation with thymidylate synthase and histopathological tumor regression after neoadjuvant 5-FU-based chemoradiotherapy. World J Gastroenterol. 2008; 14(7):1060.

Jilaveanu LB, Shuch B, Zito CR, Parisi F, Barr M, Kluger Y, et al. PD-L1 Expression in Clear Cell Renal Cell Carcinoma: An Analysis of Nephrectomy and Sites of Metastases. J Cancer. 2014; 5(3):166–72.

Kasi A, Abbasi S, Handa S, Al-Rajabi R, Saeed A, Baranda J, et al. Total Neoadjuvant Therapy vs Standard Therapy in Locally Advanced Rectal Cancer. JAMA Netw Open. 2020 Dec 16; 3(12):e2030097.

Katsumata K, Sumi T, Mori Y, Hisada M, Tsuchida A, Aoki T. Detection and evaluation of epithelial cells in the blood of colon cancer patients using RT-PCR. Int J Clin Oncol. 2006 Oct 31; 11(5):385–9.

Kaur H, Choi H, You YN, Rauch GM, Jensen CT, Hou P, et al. MR Imaging for Preoperative Evaluation of Primary Rectal Cancer: Practical Considerations. RadioGraphics. 2012 Mar; 32(2):389–409.

Kijima S. Preoperative evaluation of colorectal cancer using CT colonography, MRI, and PET/CT. World J Gastroenterol. 2014; 20(45):16964.

Kim MB, Hong TS, Wo JY. Treatment of Stage II–III Rectal Cancer Patients. Curr Oncol Rep. 2014 Jan 10; 16(1):362.

Kim S-H, Kwon H-C, Oh SY, Lee DM, Lee S, Lee J-H, et al. Prognostic Value of ERCC1, Thymidylate Synthase, and Glutathione S-Transferase π for 5-FU/Oxaliplatin Chemotherapy in Advanced Colorectal Cancer. Am J Clin Oncol. 2009 Feb; 32(1):38–43.

Labelle M, Begum S, Hynes RO. Direct Signaling between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis. Cancer Cell. 2011 Nov; 20(5):576–90.

Libutti SK, Willett CG, Saltz LB. Cancer of the rectum. In: DeVita VT Jr, Lawrence TS, Rosenberg SA. Cancer: Principles and Practice of Oncology. 9th ed. Lippincott Williams & Wilkins, 2011, pp 1127-41.

Loos M, Quentmeier P, Schuster T, Nitsche U, Gertler R, Keerl A, et al. Effect of Preoperative Radio(chemo)therapy on Long-term Functional Outcome in Rectal Cancer Patients: A Systematic Review and Meta-analysis. Ann Surg Oncol. 2013 Jun 27; 20(6):1816–28.

Low WS, Wan Abas WAB. Benchtop Technologies for Circulating Tumor Cells Separation Based on Biophysical Properties. Biomed Res Int. 2015; 2015:1–22.

Maas M, Beets-Tan RGH, Lambregts DMJ, Lammering G, Nelemans PJ, Engelen SME, et al. Wait-and-See Policy for Clinical Complete Responders After Chemoradiation for Rectal Cancer. J Clin Oncol. 2011 Dec 10; 29(35):4633–40.

Magni E, Botteri E, Ravenda PS, Cassatella MC, Bertani E, Chiappa A, et al. Detection of circulating tumor cells in patients with locally advanced rectal cancer undergoing neoadjuvant therapy followed by curative surgery. Int J Colorectal Dis. 2014 Sep 10; 29(9):1053–9.

Mamdouhi T, Twomey JD, McSweeney KM, Zhang B. Fugitives on the run: circulating tumor cells (CTCs) in metastatic diseases. Cancer Metastasis Rev. 2019 Jun 3; 38(1–2):297–305.

Martens MH, Maas M, Heijnen LA, Lambregts DMJ, Leijtens JWA, Stassen LPS, et al. Long-term Outcome of an Organ Preservation Program After Neoadjuvant Treatment for Rectal Cancer. J Natl Cancer Inst. 2016 Dec 10; 108(12):djw171.

Massagué J. TGFβ in Cancer. Cell. 2008 Jul; 134(2):215–30.

Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, et al. Frequent expression of PD-L1 on circulating breast cancer cells. Mol Oncol. 2015 Nov; 9(9):1773–82.

Mego M, Mani SA, Cristofanilli M. Molecular mechanisms of metastasis in breast cancer—clinical applications. Nat Rev Clin Oncol. 2010 Dec 19; 7(12):693–701.

Mohiuddin M, Paulus R, Mitchell E, Hanna N, Yuen A, Nichols R, et al. Neoadjuvant Chemoradiation for Distal Rectal Cancer: 5-Year Updated Results of a Randomized Phase 2 Study of Neoadjuvant Combined Modality Chemoradiation for Distal Rectal Cancer. Int J Radiat Oncol. 2013 Jul; 86(3):523–8.

Navarro M, Dotor E, Rivera F, Sánchez-Rovira P, Vega-Villegas ME, Cervantes A, et al. A Phase II study of preoperative radiotherapy and concomitant weekly irinotecan in combination with protracted venous infusion 5-fluorouracil, for resectable locally advanced rectal cancer. Int J Radiat Oncol. 2006 Sep; 66(1):201–5.

Nemeth JA, Cher ML, Zhou Z, Mullins C, Bhagat S, Trikha M. Inhibition of alpha(v)beta3 integrin reduces angiogenesis, bone turnover, and tumor cell proliferation in experimental prostate cancer bone metastases. Clin Exp Metastasis. 2003; 20(5):413-20.

Ngan SY, Burmeister B, Fisher RJ, Solomon M, Goldstein D, Joseph D, et al. Randomized Trial of Short-Course Radiotherapy Versus Long-Course Chemoradiation Comparing Rates of Local Recurrence in Patients With T3 Rectal Cancer: Trans-Tasman Radiation Oncology Group Trial 01.04. J Clin Oncol. 2012 Nov 1; 30(31):3827–33.

Okonkwo A, Musunuri S, Talamonti M, Benson A, Small W, Stryker SJ, et al. Molecular markers and prediction of response to chemoradiation in rectal cancer. Oncol Rep. 8(3):497–500.

Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev. 2008 Feb; 18(1):11–8.

Papaccio F, Roselló S, Huerta M, Gambardella V, Tarazona N, Fleitas T, et al. Neoadjuvant Chemotherapy in Locally Advanced Rectal Cancer. Cancers (Basel). 2020 Dec 3; 12(12):3611.

Park J, Yoon SM, Yu CS, Kim JH, Kim TW, Kim JC. Randomized phase 3 trial comparing preoperative and postoperative chemoradiotherapy with capecitabine for locally advanced rectal cancer. Cancer. 2011 Aug 15; 117(16):3703–12.

Paschke S, Jafarov S, Staib L, Kreuser E-D, Maulbecker-Armstrong C, Roitman M, et al. Are Colon and Rectal Cancer Two Different Tumor Entities? A Proposal to Abandon the Term Colorectal Cancer. Int J Mol Sci. 2018 Aug 30; 19(9):2577.

Petrelli F, Trevisan F, Cabiddu M, Sgroi G, Bruschieri L, Rausa E, et al. Total Neoadjuvant Therapy in Rectal Cancer. Ann Surg. 2020 Mar; 271(3):440–8.

Philips GK, Atkins M. Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies. Int Immunol. 2015 Jan 1; 27(1):39–46.

Ptok H. Oncological Outcome of Local vs Radical Resection of Low-Risk pT1 Rectal Cancer. Arch Surg. 2007 Jul 1; 142(7):649.

Rahbari NN, Aigner M, Thorlund K, Mollberg N, Motschall E, Jensen K, et al. Meta-analysis Shows That Detection of Circulating Tumor Cells Indicates Poor Prognosis in Patients With Colorectal Cancer. Gastroenterology. 2010 May; 138(5):1714-1726.e13.

Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Gastroenterol Rev. 2019; 14(2):89–103.

Recio-Boiles A, Kashyap S, Tsoris A, Babiker HM. Rectal Cancer. StatPearls. 2021.

Rödel C, Graeven U, Fietkau R, Hohenberger W, Hothorn T, Arnold D, et al. Oxaliplatin added to fluorouracil-based preoperative chemoradiotherapy and postoperative chemotherapy of locally advanced rectal cancer (the German CAO/ARO/AIO-04 study): final results of the multicentre, open-label, randomised, phase 3 trial. Lancet Oncol. 2015 Aug; 16(8):979–89.

Roh MS, Colangelo LH, O'Connell MJ, Yothers G, Deutsch M, Allegra CJ, et al. Preoperative Multimodality Therapy Improves Disease-Free Survival in Patients With Carcinoma of the Rectum: NSABP R-03. J Clin Oncol. 2009 Nov 1; 27(31):5124–30.

Romiti A, Raffa S, Di Rocco R, Roberto M, Milano A, Zullo A, et al. Circulating Tumor Cells Count Predicts Survival in Colorectal Cancer Patients. J Gastrointest Liver Dis. 2014 Sep 1; 23(3):279–84.

Rozenblit M, Huang R, Danziger N, Hegde P, Alexander B, Ramkissoon S, et al. Comparison of PD-L1 protein expression between primary tumors and metastatic lesions in triple negative breast cancers. J Immunother Cancer. 2020 Nov 25; 8(2):e001558.

Saha D, Roman C, Beauchamp RD. New Strategies for Colorectal Cancer Prevention and Treatment. World J Surg. 2002 Jul 1; 26(7):762–6.

Saigusa S, Toiyama Y, Tanaka K, Inoue Y, Mori K, Ide S, et al. Implication of programmed cell death ligand 1 expression in tumor recurrence and prognosis in rectal cancer with neoadjuvant chemoradiotherapy. Int J Clin Oncol. 2016 Oct 26; 21(5):946–52.

San Juan, Garcia-Leon, Rangel, Goetz, Chaffer. The Complexities of Metastasis. Cancers (Basel). 2019 Oct 16; 11(10):1575.

Sastre J, Maestro ML, Puente J, Veganzones S, Alfonso R, Rafael S, et al. Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. Ann Oncol. 2008 May; 19(5):935–8.

Sauer R, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, et al. Preoperative versus Postoperative Chemoradiotherapy for Rectal Cancer. N Engl J Med. 2004 Oct 21; 351(17):1731–40.

Sauer R, Liersch T, Merkel S, Fietkau R, Hohenberger W, Hess C, et al. Preoperative Versus Postoperative Chemoradiotherapy for Locally Advanced Rectal Cancer: Results of the German

CAO/ARO/AIO-94 Randomized Phase III Trial After a Median Follow-Up of 11 Years. J Clin Oncol. 2012 Jun 1; 30(16):1926–33.

Saw RPM, Morgan M, Koorey D, Painter D, Findlay M, Stevens G, et al. p53, Deleted in Colorectal Cancer Gene, and Thymidylate Synthase as Predictors of Histopathologic Response and Survival in Low, Locally Advanced Rectal Cancer Treated With Preoperative Adjuvant Therapy. Dis Colon Rectum. 2003 Feb; 46(2):192–202.

Schmoll H-J, Haustermans K, Price TJ, Nordlinger B, Hofheinz R, Daisne J-F, et al. Preoperative chemoradiotherapy and postoperative chemotherapy with capecitabine and oxaliplatin versus capecitabine alone in locally advanced rectal cancer: First results of the PETACC-6 randomized phase III trial. J Clin Oncol. 2013 May 20; 31(15_suppl):3531–3531.

Schmoll H-J, Haustermans K, Price TJ, Nordlinger B, Hofheinz R, Daisne J-F, et al. Preoperative chemoradiotherapy and postoperative chemotherapy with capecitabine and oxaliplatin versus capecitabine alone in locally advanced rectal cancer: Disease-free survival results at interim analysis. J Clin Oncol. 2014 May 20; 32(15_suppl):3501–3501.

Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. Ann Oncol. 2012 Oct; 23(10):2479–516.

Schrag D, Weiser MR, Goodman KA, Goñen M, Hollywood E, Cercek A, et al. Neoadjuvant Chemotherapy Without Routine Use of Radiation Therapy for Patients With Locally Advanced Rectal Cancer: A Pilot Trial. J Clin Oncol. 2014 Feb 20; 32(6):513–8.

Sebag-Montefiore D, Adams R, Gollins S, Samuel LM, Glynne-Jones R, Harte R, et al. ARISTOTLE: A phase III trial comparing concurrent capecitabine with capecitabine and irinotecan (Ir) chemoradiation as preoperative treatment for MRI-defined locally advanced rectal cancer (LARC). J Clin Oncol. 2020 May 20; 38(15_suppl):4101–4101.

Sharma R. An examination of colorectal cancer burden by socioeconomic status: evidence from GLOBOCAN 2018. EPMA J. 2020 Mar 11; 11(1):95–117.

Shaukat A, Mongin SJ, Geisser MS, Lederle FA, Bond JH, Mandel JS, et al. Long-Term Mortality after Screening for Colorectal Cancer. N Engl J Med. 2013 Sep 19; 369(12):1106–14.

Shen YN, Bae I-S, Park GH, Choi HS, Lee K-H, Kim SH. MicroRNA-196b enhances the radiosensitivity of SNU-638 gastric cancer cells by targeting RAD23B. Biomed Pharmacother. 2018 Sep; 105:362–9.

Shi D, Yang C, Han S, Wang S, Xiong B. Dynamic evaluation of mesenchymal circulating tumor cells in patients with colorectal cancer: Clinical associations and prognostic value. Oncol Rep. 2020 May 29; 44(2):757–67.

Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? Curr Opin Immunol. 2015 Apr; 33:23–35.

Silva VS e, Abdallah EA, Brito ABC de, Braun AC, Tariki MS, de Mello CAL, et al. Baseline and Kinetic Circulating Tumor Cell Counts Are Prognostic Factors in a Prospective Study of Metastatic Colorectal Cancer. Diagnostics. 2021 Mar 12; 11(3):502.

Smith FM, Rao C, Perez RO, Bujko K, Athanasiou T, Habr-Gama A, et al. Avoiding Radical Surgery Improves Early Survival in Elderly Patients With Rectal Cancer, Demonstrating Complete Clinical Response After Neoadjuvant Therapy. Dis Colon Rectum. 2015 Feb; 58(2):159–71.

Souza e Silva V, Chinen L, Abdallah E, Damascena A, Paludo J, Chojniak R, et al. Early detection of poor outcome in patients with metastatic colorectal cancer: tumor kinetics evaluated by circulating tumor cells. Onco Targets Ther. 2016 Dec; 9:7503–13.

Stoecklein NH, Hosch SB, Bezler M, Stern F, Hartmann CH, Vay C, et al. Direct Genetic Analysis of Single Disseminated Cancer Cells for Prediction of Outcome and Therapy Selection in Esophageal Cancer. Cancer Cell. 2008 May; 13(5):441–53.

Sun W, Al-Rajabi R, Perez RO, Abbasi S, Ash R, Habr-Gama A. Controversies in Rectal Cancer Treatment and Management. Am Soc Clin Oncol Educ B. 2020 May; (40):136–46.

Sun W, Li G, Wan J, Zhu J, Shen W, Zhang Z. Circulating tumor cells: A promising marker of predicting tumor response in rectal cancer patients receiving neoadjuvant chemo-radiation therapy. Oncotarget. 2016 Oct 25; 7(43):69507–17.

Thiery JP. Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002 Jun; 2(6):442–54.

Tol J, Koopman M, Miller MC, Tibbe A, Cats A, Creemers GJM, et al. Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. Ann Oncol. 2010 May; 21(5):1006–12.

Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol. 2012 Apr; 24(2):207–12.

Tsai W-S, Chen J-S, Shao H-J, Wu J-C, Lai J-M, Lu S-H, et al. Circulating Tumor Cell Count Correlates with Colorectal Neoplasm Progression and Is a Prognostic Marker for Distant Metastasis in Non-Metastatic Patients. Sci Rep. 2016 Apr 14; 6(1):24517.

Tseng M, Soon YY, Vellayappan B, Ho F, Tey J. Radiation therapy for rectal cancer. J Gastrointest Oncol. 2019 Dec; 10(6):1238–50.

Tuta M, Boc N, Brecelj E, Omejc M, Anderluh F, Ermenc AS, et al. Total neoadjuvant treatment of locally advanced rectal cancer with high risk factors in Slovenia. Radiol Oncol. 2019 Oct 25; 53(4):465–72.

Tuta M, Boc N, Brecelj E, Peternel M, Velenik V. Total neoadjuvant therapy vs standard therapy of locally advanced rectal cancer with high-risk factors for failure. World J Gastrointest Oncol. 2021 Feb 15;13(2):119–30.

Vaddepally RK, Kharel P, Pandey R, Garje R, Chandra AB. Review of Indications of FDA-Approved Immune Checkpoint Inhibitors per NCCN Guidelines with the Level of Evidence. Cancers (Basel). 2020 Mar 20; 12(3):738.

Van der Toom EE, Verdone JE, Gorin MA, Pienta KJ. Technical challenges in the isolation and analysis of circulating tumor cells. Oncotarget. 2016 Sep 20; 7(38):62754–66.

Van der Valk MJM, Hilling DE, Bastiaannet E, Meershoek-Klein Kranenbarg E, Beets GL, Figueiredo NL, et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. Lancet. 2018 Jun; 391(10139):2537–45.

Vatandoust S. Colorectal cancer: Metastases to a single organ. World J Gastroenterol. 2015; 21(41):11767.

Vecchio FM, Valentini V, Minsky BD, Padula GDA, Venkatraman ES, Balducci M, et al. The relationship of pathologic tumor regression grade (TRG) and outcomes after preoperative therapy in rectal cancer. Int J Radiat Oncol. 2005 Jul; 62(3):752–60.

Wang J, Li S, Liu Y, Zhang C, Li H, Lai B. Metastatic patterns and survival outcomes in patients with stage IV colon cancer: A population-based analysis. Cancer Med. 2020 Jan 6; 9(1):361–73.

Willeke F, Horisberger K, Kraus-Tiefenbacher U, Wenz F, Leitner A, Hochhaus A, et al. A phase II study of capecitabine and irinotecan in combination with concurrent pelvic radiotherapy (CapIri-RT) as neoadjuvant treatment of locally advanced rectal cancer. Br J Cancer. 2007 Mar 27 ;96(6):912–7.

Yeo S-G, Kim DY, Kim TH, Chang HJ, Oh JH, Park W, et al. Pathologic Complete Response of Primary Tumor Following Preoperative Chemoradiotherapy for Locally Advanced Rectal Cancer. Ann Surg. 2010 Dec; 252(6):998–1004.

Yoshimura K, Inoue Y, Karayama M, Tsuchiya K, Mori K, Suzuki Y, et al. Heterogeneity analysis of PD-L1 expression and copy number status in EBUS-TBNA biopsy specimens of non-small cell lung cancer: Comparative assessment of primary and metastatic sites. Lung Cancer. 2019 Aug; 134:202–9.

Zam W, Ali L. Immune checkpoint inhibitors in the treatment of cancer. Curr Clin Pharmacol. 2021 Mar 25; 16.

Zarzynska JM. Two Faces of TGF-Beta1 in Breast Cancer. Mediators Inflamm. 2014; 2014:1–16.

Zhang J, Qiao X, Shi H, Han X, Liu W, Tian X, et al. Circulating tumor-associated neutrophils (cTAN) contribute to circulating tumor cell survival by suppressing peripheral leukocyte activation. Tumor Biol. 2016 Apr 12; 37(4):5397–404.

Zhao G, Deng L, Ye D, Wang W, Yan Y, Yu T. Efficacy and safety of wait and see strategy versus radical surgery and local excision for rectal cancer with cCR response after neoadjuvant chemoradiotherapy: a meta-analysis. World J Surg Oncol. 2020 Dec 31; 18(1):232.

Anexo 1 - Carta de aprovação do Comitê de Ética em Pesquisa (CEP)

A.C.Camargo Cancer Center
Centro Integrado de Diagnóstico, Tratamento, Ensino e Pesquisa

COMITÊ DE ÉTICA
EM PESQUISA - CEP

São Paulo, 26 abril de 2017.

À
Dra. Ludmilla Thomé Domingos Chinen
Aluno: Virgilio Souza e Silva (Doutorado)

Ref.: Projeto de Pesquisa nº. 2141/15D
"Câncer de Reto Localmente Avançado: Identificação e Correlação Molecular e Dinâmica de Proteínas Relacionadas à Resistência ao Tratamento Neoadjuvante e Adjuvante à Resposta Imune em Células Tumorais Circulantes."

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – A.C. Camargo Cancer Center, em reunião de **04/04/2017**, **tomaram conhecimento e aprovaram** os seguintes documentos:

- Solicitação de dispensa da submissão da documentação obrigatória e análise ética do projeto acima mencionado por se tratar de um projeto afiliado ao temático intitulado "Identificação de Proteínas e Genes de Fatores de Transcrição Relacionados à Resistência a Quimioterapia e Radioterapia em Células Tumorais Circulantes de Pacientes com Câncer de Cólon e Reto Localmente Avançados", registrado neste CEP sob nº 2141/15. O projeto afiliado em referência será Doutorado do aluno *Virgilio Souza e Silva;*
- Tese de Doutorado.

Atenciosamente,

Dra. Sandra Caires Serrano
2ª. Vice-Coordenadora do Comitê de Ética em Pesquisa

1/1

Anexo 2 - Carta de aprovação do Comitê de Ética em Pesquisa (CEP) de conhecimento da modificação do título do projeto.

A.C.Camargo Cancer Center
Centro Integrado de Diagnóstico, Tratamento, Ensino e Pesquisa

COMITÊ DE ÉTICA
EM PESQUISA - CEP

São Paulo, 22 de julho de 2020.

Á
Dra. Ludmilla Thomé Domingos Chinen
Aluno: Virgilio Souza e Silva (Doutorado)

Ref.: Projeto de Pesquisa nº. 2141/15D
"Câncer de Reto Localmente Avançado: Identificação e Correlação Molecular e Dinâminca de Proteínas Relacionadas à Resistência ao Tratamento Neoadjuvante e Adjuvante à Resposta Imune em Células Tumorais Circulantes"

Os membros do Comitê de Ética em Pesquisa da Fundação Antonio Prudente – A.C. Camargo Cancer Center, em sua última reunião de **14/07/2020, tomaram conhecimento** do seguinte documento:

- Solicitação de mudança de título do projeto em referência para: "IDENTIFICAÇÃO E CORRELAÇÃO MOLECULAR E DINÂMICA DE PROTÉINAS RELACIONADAS À RESISTÊNCIA AO TRATAMENTO NEOADJUVANTE EM CÉLULAS TUMORAIS CIRCULANTES DE PACIENTES COM CÂNCER DE RETO LOCALMENTE AVANÇADO", em documento datado de 04 de junho de 2020.

Atenciosamente,

Luciana Facure Moredo
1ª. Vice-Coordenadora do Comitê de Ética em Pesquisa

Anexo 3 - Artigo 1 publicado.

Revista Cells. *Cells* **2019**, *8*(7), 641; **https://doi.org/10.3390/cells8070641**

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Article

Molecular and Kinetic Analyses of Circulating Tumor Cells as Predictive Markers of Treatment Response in Locally Advanced Rectal Cancer Patients

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Abstract: Neoadjuvant chemoradiation (NCRT) followed by total mesorectal excision is the standard treatment for locally advanced rectal cancer (LARC). To justify a non-surgical approach, identification of pathologic complete response (pCR) is required. Analysis of circulating tumor cells (CTCs) can be used to evaluate pCR. We hypothesize that monitoring of thymidylate synthase (TYMS) and excision repair protein, RAD23 homolog B (RAD23B), can be used to predict resistance to chemotherapy/radiotherapy. Therefore, the aims of this study were to analyze CTCs from patients with LARC who underwent NCRT plus surgery for expression of TYMS/RAD23B and to evaluate their predictive value. Blood samples from 30 patients were collected prior to NCRT (S1) and prior to surgery (S2). CTCs were isolated and quantified by ISET®, proteins were analyzed by immunocytochemistry, and *TYMS* mRNA was detected by chromogenic in situ hybridization. CTC counts decreased between S1 and S2 in patients exhibiting pCR ($p = 0.02$) or partial response ($p = 0.01$). Regarding protein expression, TYMS was absent in 100% of CTCs from patients with pCR ($p = 0.001$) yet was expressed in 83% of non-responders at S2 ($p < 0.001$). Meanwhile, RAD23B was expressed in CTCs from 75% of non-responders at S1 ($p = 0.01$) and in 100% of non-responders at S2 ($p = 0.001$). Surprisingly, 100% of non-responders expressed *TYMS* mRNA at both timepoints ($p = 0.001$). In addition, TYMS/RAD23B was not detected in the CTCs of patients exhibiting pCR ($p = 0.001$). We found 83.3% of sensitivity for TYMS mRNA at S1 ($p = 0.001$) and 100% for TYMS ($p = 0.064$) and RAD23B ($p = 0.01$) protein expression at S2. Thus, *TYMS* mRNA and/or TYMS/RAD23B expression in CTCs, as well as CTC kinetics, have the potential to predict non-response to NCRT and avoid unnecessary radical surgery for LARC patients with pCR.

Keywords: locally advanced rectal cancer; circulating tumor cells; RAD23B; thymidylate synthase; chemoradioresistance

1. Introduction

Colorectal carcinoma is one of the most commonly occurring neoplasms in the Western world. Moreover, despite improvements in treatment, colorectal carcinoma remains an important cause of

cancer-related deaths worldwide [1]. Rectal carcinoma, which accounts for approximately 30% of all primary colorectal cancers, is characterized by an anatomy and natural history that are distinct from other colonic tumors [2,3].

Neoadjuvant chemoradiation (NCRT) followed by total mesorectal excision is the standard treatment for locally advanced rectal carcinoma (LARC). NCRT is generally recommended for patients with cT3/T4 disease or lymph node involvement [4].

The absence of viable tumor cells in surgical specimens after NCRT is defined as pathological complete response (pCR). It occurs in 10–30% of patients [5] and it is associated with a better prognosis. Capecitabin and 5-fluorouracil (5-FU) are currently the most widely used radiosensitizers for NCRT. In some patients, chemoradiation induces pCR, although radical surgery is recommended for most patients [6,7].

Accurate identification of patients with pCR is necessary before pursuing a non-surgical approach. Many studies have evaluated the accuracy of the digital rectal exam and images in identifying patients with pCR [8,9]. However, the sensitivity and specificity of these methods are too low to accurately predict pCR. Molecular analyses of gene expression have also been performed and have been shown to be unsuitable for identifying patients with pCR who can be followed without radical surgery [10]. Recently, promising data have supported the use of a "watch and wait" strategy for patients who have no signs of a viable tumor in a digital rectal exam, rectoscopy (with or without biopsy), or imaging. The likelihood of tumor regrowth is minimal for these patients, and most could be cured by salvage surgery [11].

In addition, considering that the majority of patients will not respond to NCRT, it is extremely important to identify these patients, in an attempt to optimize the response to these patients. As there are no randomized phase-III studies to date, comparing this strategy with isolated induction chemoradiotherapy, it becomes fundamental to identify a biomarker capable of selecting patients for these approach [12–14].

For organ-preserving treatment strategies, the ability to identify tissues or blood biomarkers that predict NCRT response/non-response is very important. Circulating tumor cells (CTCs) are a real-time source of biomarkers, which have shown promise in facilitating the detection and monitoring of pCR and non-responder patients. It has been proposed that the identification and analysis of CTCs would facilitate investigations to understand intrinsic tumor features and characteristics of patients with pCR or non-responders so that individualized treatment strategies can be applied [15]. To date, use of CTC counts has been approved by the US Food and Drug Administration as a prognostic tool for metastatic prostate, colon, and breast cancers [16–18]. Additionally, CTC kinetics can be used to monitor tumor response to systemic treatment [19,20]. Meanwhile, molecular characterization of CTCs has facilitated studies of biomarkers, including proteins, gene expression, and chromosomal translocations [21,22].

Since only a small proportion of patients with LARC experience complete response following chemoradiation, an improved understanding of the molecular mechanisms underlying resistance to chemotherapy and radiation therapy resistance is essential. For chemotherapy, the main target of 5-FU is the enzyme thymidylate synthase (TYMS). TYMS expression analysis has been used to predict individual response to NCRT and has exhibited good prognostic value for rectal cancer recurrence [23,24]. Radiation therapy is an effective component of neoadjuvant treatment and it induces genetic damage. Consequently, the ultraviolet excision repair protein, RAD23 homolog B (RAD23B), which is part of the nucleotide excision repair process [25,26], would potentially be induced by the genetic damage introduced by radiation therapy. Recently, RAD23B was found to be associated with breast cancer relapse risk [27].

The aim of the present study was to explore the role of CTCs in patients undergoing NCRT followed by surgery for treatment of LARC. In addition, the predictive values of TYMS and RAD23B before and after NCRT were evaluated.

2. Methods

2.1. Patients and Treatment

This prospective study was conducted at the A. C. Camargo Cancer Center (São Paulo, Brazil) and was approved by the local ethics committee (2141/15C). Written informed consent was obtained from all patients prior to enrolment. Patients met the inclusion criteria for this study if they had a diagnosis of rectal cancer, as confirmed by biopsy pathology; had locally advanced disease staged as cT3–cT4 or N0–N+; and were candidates for chemoradiation therapy per institutional protocol. Patients were excluded if they had evidence of distant metastasis; a history of any surgery (e.g., colostomy) within two weeks prior to the initiation of treatment; or were taking anticoagulants at the time of the study. Cancer stage was determined with pelvic magnetic resonance imaging and chest and abdominal computed tomography.

Blood samples were collected at baseline or prior to NCRT (S1), and then prior to surgery (S2). Venous blood was collected from the antecubital vein and these samples were stored at room temperature for a maximum of 4 h prior to analysis.

Radiation therapy was applied with a three-dimensional (3D) conformal technique. A 45-Gy dose was applied in 25 fractions over the entire pelvis. In addition, a 5.4-Gy radiation boost was administered to the primary tumor and involved lymph nodes in three fractions, for a total of 50.4 Gy applied in 28 fractions. Chemotherapy regimens consisted of either intravenous 5-FU administered at a dose of 1000 mg/m^2 on days 1–5 during weeks 1 and 5 of radiation therapy; or oral capecitabin administered at a dose of 1650 mg/m^2/d during the entire radiation treatment period. Each patient's physician determined which regimen was appropriate.

The evaluation of the response was determined by the comparative analysis of the baseline images in S1 with the images before surgery (S2). In addition, we evaluated the pathologic response in comparison to the clinical staging established by baseline images.

2.2. Isolation and Quantification of CTCs and Protein Analysis of TYMS and RAD

ISET® (Isolation by size of epithelial tumors) was used to quantify and analyze CTCs. Briefly, peripheral blood samples were collected from patients into EDTA tubes (8.0 mL BD Vacutainer, Franklin Lakes, NJ, USA) and then were homogenized at room temperature for up to 4 h. Samples were then prepared as described previously [28]. Briefly, ISET membrane spots were cut out and subjected to immunocytochemistry (ICC) with an anti-RAD23B antibody (1:100 CSB-PA019260LA01HU; CusaBio, Wuhan, People's Republic of China), an anti-TYMS antibody (1:230 WH0007298M1; Sigma-Aldrich, St. Louis, MO, USA), and an anti-CD45 antibody (1:200 HPA000440; Sigma-Aldrich).

Selected spots from the ISET membranes were additionally subjected to a 24-well dual color ICC assay (Polink DS-RR-Hu/Ms A Kit; GBI Labs, Bothell, WA, USA). Briefly, antigen retrieval was performed by using a DakoTM Antigen Retrieval Solution (Dako, Santa Clara, CA, USA). Cells were then hydrated with 1X Tris-buffered saline (TBS) for 10 min and then permeabilized with Triton X-100 for 5 min. Next, cells were rinsed with 1X TBS and incubated with 3% hydrogen peroxide in the dark for 15 min to block endogenous peroxides. Immobilized cells on the membrane spots were incubated overnight with primary antibodies diluted in 10% fetal calf serum in TBS. To amplify primary antibody signals, the spots were incubated for 30 min with rabbit horseradish peroxidase (HRP) polymer (GBI Labs), then with 3,3-diaminobenzidine (DAB) for 10 min. After amplification, the spots were incubated with a second primary antibody for 2 h, a rabbit AP polymer (GBI Labs) for 30 min, and then GBI-Permanent Red (GBI Labs) for 10 min. The latter reagent was freshly prepared according to the manufacturer's instructions. After staining the cells with haematoxylin, specimens were examined with light microscopy (Research System Microscope BX61; Olympus, Waltham, MA, USA). CTCs were counted per 1 mL blood, as previously described by Krebs et al. [29]. CTCs were characterized according to five criteria: Negativity for CD45 staining; nucleus size >12 µm, hyperchromatic and irregular nuclei; visible cytoplasm; and a nuclear to cytoplasm ratio >80% [30]. CTCs were considered

positive for TYMS or RAD23B expression if at least one cell in a specimen stained for these markers on ICC analysis.

2.3. CTC Isolation and Immunostaining Control

Negative controls were healthy donor blood filtered by ISET®. Positive controls included healthy donor blood spiked with HCT-8 colorectal carcinoma cells. For the ICC reaction and TYMS antibody control, leukocytes from healthy filtered blood were used. According to the Human Protein Atlas (http://www.proteinatlas.org/), the latter express TYMS. As a positive control for the RAD23B antibody, HCT-8 cells were spiked in healthy donor blood and filtered on ISET®. To create a negative control for ICC, the same cell line was used without primary antibodies in order to ensure exclusion of cross-reactivity. To confirm that analyzed CTCs were not leukocytes, staining with an anti-CD45 antibody was performed.

2.4. Chromogenic In situ Hybridization Assay for TYMS

TYMS mRNA was detected in intact cells by using a chromogenic in situ hybridization (CISH) assay employing RNAscope Technology (ACDbio, Newark, CA, USA), according to the manufacturer's protocol. Cytology methods standardized by our group were also used. Briefly, one spot was cut out for each patient sample and these were placed in individual wells of a 24-well plate. The membrane spots were hydrated with 1X TBS for 5 min before being incubated in 1% formaldehyde solution for 5 min at room temperature. The spots were then rinsed 2× with distilled water before applying 5–8 drops of RNAscope Hydrogen Peroxide (ACDbio) to each spot. After incubating the samples in a humid chamber for 10 min at room temperature, the spots were washed 2× with distilled water and mounted on slides. To each slide, 3 drops of cytology pepsin were applied. After 10 min at room temperature the spots were returned to the 24-well plate, washed 2× with distilled water, then rapidly dehydrated in successive 1-min incubations with 70%, 85%, and 100% ethanol solutions. After the spots were left to air dry on the slides, 3 drops of a TYMS-specific probe were added to each spot. After 2 h at 40 °C in a HybEZ oven hybridizer (ACDbio), drops of Amp1-6 solutions were added, according to the manufacturer's protocol, with washes performed with 1X TBS. Finally, the cells were incubated with chromogen and 50% haematoxylin then placed on slides with aqueous mounting media and coverslips. The samples were inspected with brightfield microscopy (BX61-Olympus; Olympus). To classify *TYMS* mRNA expression, an absence or presence of staining was classified as negative or positive, respectively.

2.5. Statistical Analysis

Initially, a descriptive analysis was performed to obtain absolute (n) and relative (%) frequency distributions. To evaluate possible associations between the variables of interest, a contingency table was constructed from sample data. Chi-square tests of independence or Fisher's exact test were used, as appropriate. The level of significance was set at 5% and all statistical analyses were performed by using the SPSS program for Windows, version 25.

3. Results

3.1. Patients

Thirty patients with rectal cancer were enrolled in this study between April 2016 and January 2018. Clinical characteristics of these patients are listed in Table 1. The mean age of this cohort was 56 years (range, 34–72) and 60% of the patients were male. For 67% of the patients, their rectal tumors were located 7 cm or less from the anal verge.

The baseline (prior to NCRT) T stage was cT2 for 4 patients (13%), cT3 for 21 patients (70%), and cT4 for 5 patients (17%). Baseline N stage was cN0 for 22 patients (73%) and cN+ for 8 patients (27%). The mean time to surgery completion following NCRT was 77 days. Pathologic T stage was

pT0 for 6 patients (20%), pT1 for 5 patients (17%), pT2 for 7 patients (23%), pT3 for 9 patients (30%), and pT4 for 2 patients (7%). Pathologic N stage was pN0 for 21 patients (70%), pN1 for 6 patients (20%), and pN2 for 2 patients (7%). One patient (3%) exhibited disease progression during NCRT. Complete pathologic response after preoperative therapy was detected in 6 patients (20%), while 7 patients (23%) had their tumors down-staged (ypT1-2N0).

Table 1. Patient characteristics.

Characteristics	*N* (%)
Average age (min–max), years	56 (34–72)
Gender	
Male	18 (60)
Female	12 (40)
Tumor distance from the anal verge	
≤7 cm	20 (67)
>7 cm	10 (33)
Clinical T baseline stage	
T2	04 (13)
T3	21 (70)
T4	05 (17)
Clinical N baseline stage	
N0	22 (73)
N+	08 (27)
Pathological T stage	
T0	06 (20)
T1–T2	12 (40)
T3–T4	11 (37)
DP	1 (3)
Pathological N stage	
N0	21 (70)
N1–N2	08 (27)
DP	01 (3)
Average time (min–max) of completion of RDT for surgery (days)	77 (50–143)

Abbreviations: DP: Disease Progression; RDT: radiotherapy.

All patients were treated with 25 fractions of 45 Gy to the pelvis with a 3D conformal technique. In addition, a 5.4-Gy radiation boost was applied to the primary tumor. There were no treatment delays or interruptions that lasted more than two days. Twenty-six patients (86.7%) received intravenous 5-FU and four patients (13.3%) received capecitabine. The main treatment toxicities were grade 1 or 2 diarrhea in 10 patients (33%) and grade 1 or 2 oral mucositis in 8 patients (27%). No grade 3 or 4 toxicities were reported. There were also no differences in adverse events between the two chemotherapy groups.

3.2. CTCs

CTCs were detected at S1 in all 30 patients of our cohort, and at S2 in 24 patients (Figure 1A,D). The mean CTC concentrations were 6 cells/mL at S1 and 3.5 cells/mL at S2. Kinetic analyses showed that CTC levels were increased in 3 patients, decreased in 22 patients, and remained unchanged in 5 patients. The mean CTC count per mL was 3.1 for those with pCR, 2.5 for those exhibiting a partial response (PR), and 2.9 for those with non-responsive tumors. Patients exhibiting pCR and PR showed a decrease in CTC kinetics (calculated as: CTC baseline [CTC1] × CTC post-CRT [CTC2]) during treatment ($P = 0.02$ and $P = 0.01$, respectively; Table 2).

Table 2. Kinetic counts of circulating tumor cells (CTCs) between baseline (CTC1) and post-neoadjuvant chemoradiation (NCRT) (CTC2) time points.

9	Patient ID	CTCs/mL before NCRT	CTCs/mL after NCRT	Kinetics of CTC1 vs. CTC2	
CR	8	3	1	>	P = 0.02
	11	4	1	>	
	18	1	0	>	
	21	4	0	>	
	25	4	2	>	
	27	3	2	>	
PR	3	5	5	=	P = 0.01
	4	3	2	>	
	7	3	2	>	
	9	1	0	>	
	10	0	1	<	
	13	1	1	=	
	15	2	0	>	
	16	2	0	>	
	23	2	1	>	
	24	6	3	>	
	29	3	0	>	
	30	2	2	=	
NR	1	3	4	<	P = 0.07
	2	3	2	>	
	5	1	1	=	
	6	1	1	=	
	12	1	4	<	
	14	2	1	>	
	17	3	1	>	
	19	4	2	>	
	20	5	1	>	
	22	8	4	>	
	26	2	1	>	
	28	2	1	>	

Abbreviations: NCRT: Neoadjuvant chemoradiotherapy; CR: Complete response; PR: Partial response; NR: No response.

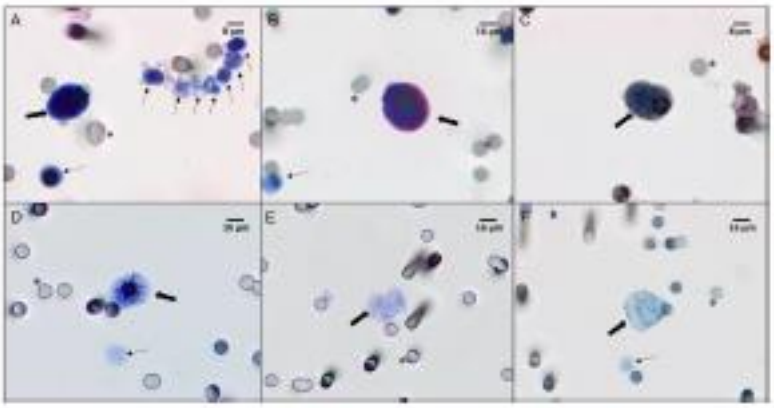

Figure 1. Immunostaining (A–C) and chromogenic in situ hybridization (CISH) (D–F) of CTCs from locally advanced rectal cancer (LARC) patients. (A) CTCs and leukocytes visualized with haematoxylin-eosin staining (×40 magnification). (B) CTCs stained with an anti-thymidylate synthase (TYMS) antibody, visualized with Permanent Red, and counterstained with haematoxylin (×40 magnification). (C) CTCs stained with an anti-RAD23B antibody, visualized with 3,3-diaminobenzidine (DAB), and counterstained with haematoxylin (×60 magnification). (D) CTCs negative for *TYMS* mRNA and counterstained with haematoxylin (×40 magnification). (E) CTCs with a low *TYMS* mRNA signal (normal expression) counterstained with haematoxylin (×40 magnification). (F) CTCs with a high *TYMS* mRNA signal (overexpression) counterstained with haematoxylin (×40 magnification). All images were analyzed on a Research System Microscope BX61 (Olympus, Tokyo, Japan) coupled to a digital camera (SC100-Olympus). Thick arrows indicate CTCs, thin arrows indicate leukocytes, and asterisks indicate 8 µm pores of the ISET® membranes.

3.3. *TYMS* and *RAD23B*

At baseline, TYMS-positive CTCs were detected in 7 patients (23.5%) by ICC (Figure 1B) and in 21 patients (70%) by CISH (Figure 1E,F). RAD23B-positive CTCs were detected in 13 patients (43.3%) by ICC (Figure 1C).

After chemoradiation, TYMS-positive CTCs were detected in 10 patients (41.6%) by ICC, while *TYMS* mRNA was detected in 16 patients (61.5%) by CISH. RAD23B-positive CTCs were detected in 14 patients (58.3%).

Baseline *TYMS* mRNA and RAD23B-positive CTCs were associated with poor clinical response. For example, all 12 patients with non-responsive tumors had *TYMS* mRNA detected in their CTCs by CISH. In contrast, 83.5% of patients with pCR did not have CTCs expressing *TYMS* mRNA ($P = 0.001$; Table 3). At S1, RAD23B-positive CTCs were detected in 33% of patients with pCR, in 16% of patients with PR, and in 75% of patients exhibiting no response ($P = 0.01$). Thus, there was no association between detection of TYMS expression in CTCs by ICC and response type in S1 (Table 3). To confirm the value of these proteins as biomarkers, we compared these found with the most used clinical parameter, which is pathological response of the primary tumor. We found, for TYMS mRNA, 83.3% sensitivity, 83.3% specificity, and 95.2% positive predictive value (PPV) ($p = 0.001$) (supplementary table).

Table 3. Expression profiles of RAD23B and TYMS proteins and *TYMS* mRNA.

			CR	*PR*	*NR*	
Before NCRT	CISH (TYMS)	+	16.5	66.5	100	P = 0.001
		−	83.5	33.5	0	
	TYMS (protein)	+	16.5	25	25	P = 1.0
		−	83.5	75	75	
	RAD (protein)	+	33.5	16.5	75	P = 0.01
		−	66.5	83.5	25	
After NCRT	CISH (TYMS)	+	25	30	100	P = 0.001
		−	75	70	0	
	TYMS (protein)	+	0	0	83.5	P = 0.0001
		−	100	100	16.5	
	RAD (protein)	+	0	25	100	P = 0.0001
		−	100	75	0	

Abbreviations: NCRT: Neoadjuvant chemoradiotherapy; CR: Complete response; PR: Partial response; NR: No response.

Post-chemoradiation analyses (S2) showed that expression of TYMS and RAD23B in CTCs strongly correlates with poor response. For example, TYMS-positive CTCs were not detected in the patients with pCR or pPR, yet they were detected in 83% of non-responsive patients ($P < 0.001$; Table 3). Furthermore, *TYMS* mRNA expression at S2 correlated with response, with *TYMS* mRNA detected in all of the patients exhibiting no response ($P = 0.001$). We found, for TYMS protein, 100% sensitivity, 50% specificity, and 100% positive predictive value (PPV) ($p = 0.064$). For RAD23B, the values were: 100% sensitivity, 70% specificity, and 100% positive predictive value (PPV) ($p = 0.01$) (supplementary table).

Lastly, we found that expression of TYMS and RAD23B in CTCs was strongly predictive of response type. For example, after NCRT, $TYMS^-/RAD23B^-$ CTCs were detected in 100% of the pCR patients, in 83.5% of the PR patients, and in none of the NR patients. However, $TYMS^+/RAD23B^+$ CTCs were not detected in patients with pCR or PR, and yet were detected in 83.5% of patients with no response ($P = 0.001$). At S1, CTCs expressing TYMS and RAD23B did not correlate with response type ($P = 0.1$; Table 4). Meanwhile, expression of *TYMS* mRNA determined which patients did not respond to chemoradiotherapy at S1 and S2 (Figure 2).

Table 4. Correlation between RAD and TYMS protein expression profiles.

Profile	CR %	PR %	NR %	
TYMS−/RAD−	50	66.5	16.5	Before NCRT $P = 0.1$
TYMS+/RAD+	0	8.5	16.5	
TYMS+/RAD− TYMS−/RAD+	50	25	67	
TYMS−/RAD−	100	83.5	0	After NCRT $P = 0.001$
TYMS+/RAD+	0	0	83.5	
TYMS+/RAD− TYMS−/RAD+	0	16.5	16.5	

Abbreviations: NCRT: Neoadjuvant chemoradiotherapy; CR: Complete response; PR: Partial response; NR: No response.

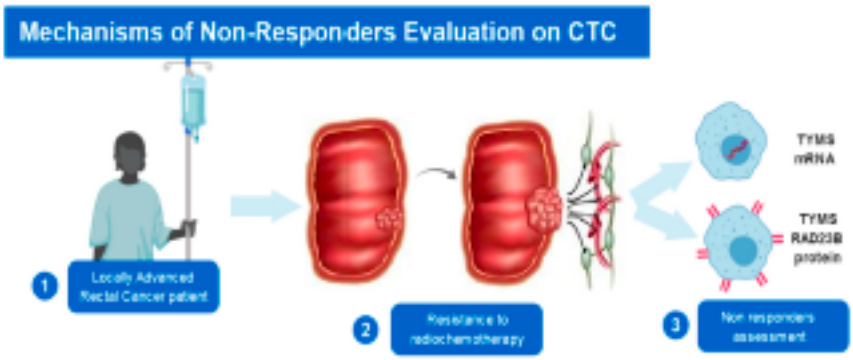

Figure 2. Scheme demonstrating both methodologies used to select responders and non-responders to neoadjuvant chemoradiotherapy: mRNA detected by CISH and protein expression detected by immunocytochemistry (ICC).

4. Discussion

LARC treatment generally consists of NCRT followed by total mesorectal excision. For high-risk patients, postoperative adjuvant chemotherapy may additionally be considered [31]. The ability to identify patients who have undergone complete eradication of a tumor following NCRT is crucial. In the current study, we prospectively demonstrated a strong correlation between expression of TYMS and RAD23B by CTCs in patients with LARC and pCR following NCRT.

Among the 50–60% of patients with LARC who respond to NCRT (i.e., their tumors are down-staged following treatment), many exhibit improved survival. It has been reported that pCR after NCRT is associated with improved cancer outcome and significantly decreased rates of local recurrence. Conversely, the 40% non-response rate after NCRT [31] represents heterogeneity in response to standard treatment [32,33]. For the latter, new therapeutic strategies, avoidance of toxicity associated with ineffective treatment, and novel neoadjuvant chemotherapeutic options are needed [34,35]. In addition, the identification of biomarkers would facilitate the creation of individualized treatment plans.

The ability to identify tumors that do not respond to radiotherapy is useful for helping patients avoid radiation side effects such as fibrosis, fecal and bladder incontinence, diarrhea, dysuria, and myelosuppression [36,37]. This knowledge would also facilitate discussions regarding alternate approaches, such as more potent neoadjuvant multi-agent chemotherapy strategies or a rationale for foregoing radiation therapy. There are several studies that have discussed these considerations, although robust biomarkers that will predict non-responders of NCRT with high accuracy still need to be identified [37–39]. In the present study, we were able to identify NCRT non-responders at S1 by detecting *TYMS* mRNA. Thus, it is possible that detection of *TYMS* mRNA in CTCs could represent a valuable tool in identifying non-responder patients prior to the start of NCRT. Furthermore, detection of

RAD23B expression could make this patient selection process more accurate. It is well-established that RAD23B is involved in DNA repair following radiation damage, and its identification in CTCs can guide treatment plans. In the present study, 75% of non-responding tumors expressed this protein on CTCs before NCRT. Furthermore, when both RAD23B and TYMS protein expression on CTCs were detected after NCRT, 100% of patients with pCR did not express either protein. Meanwhile, 83.5% of patients with non-responsive tumors expressed both proteins and the remaining 16.5% expressed at least one of these proteins. In addition, *TYMS* mRNA expression after NCRT showed high positivity for non-responders (100%) and was not related to protein expression (Figure 3). For the seven patients who presented discordant mRNA/protein positivity at S1 (Supplementary Table S1), they exhibited a correspondence between *TYMS* gene expression and TYMS protein expression at S2. Chemotherapeutic agents have previously been associated with changes in gene expression. In addition, post-transcriptional mechanisms for blocking protein synthesis have been characterized [40–42]. For 5-FU, there are several papers that describe this correlation [43,44]. The present results support the hypothesis that CTC analysis can be a useful tool for identifying patients who will respond to chemoradiotherapy. As a result, a "watch and wait" strategy becomes an option to be considered in addition to radical surgery.

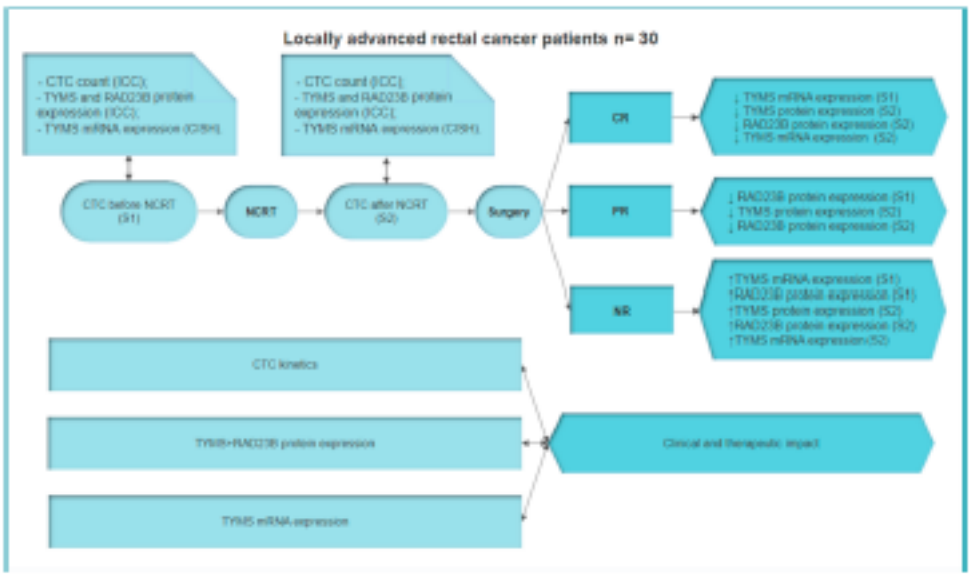

Figure 3. Summary of methodologies, analyses, and results in the present study. Patients were enrolled prior to the start of NCRT. Blood was collected to perform CTC counts and molecular analyses. At baseline, complete response (CR) correlated with low levels of *TYMS* mRNA. In contrast, NR correlated with high levels of *TYMS* mRNA and RAD23B protein expression. Blood samples were collected again during follow-up after NCRT. CTC analyses showed that CR correlated to low levels of *TYMS* mRNA and RAD23B protein expression, while NR correlated to high levels of *TYMS* mRNA and RAD23B/TYMS protein expression. CTC kinetics also correlated to pathological response. Based on these results, *TYMS* mRNA and RAD23B and TYMS protein expression appear to have a clinical and therapeutic impact in LARC patients. Abbreviations: CR: Complete response; LARC: Locally advanced rectal cancer; NCRT: Neoadjuvant chemoradiotherapy; NR: No response; PR: Partial response.

Our analysis of RAD23B and TYMS expression showed similar profiles for these two proteins. Based on the different protein patterns for TYMS in relation to pCR and PR at S1 and S2, we decided to further examine the mRNA expression of *TYMS* with RNA hybridization assays performed in situ with CTCs. All of the CTCs from non-responding patients expressed *TYMS* mRNA at both S1 and

S2. Thus, this assay exhibited high sensitivity and specificity for identifying LARC patients who are predicted to be non-responders to radiochemotherapy.

After NCRT, TYMS expression exhibited a strong correlation with *TYMS* mRNA (kappa value 0.6; $P = 0.003$) in absence of response to treatment. This second assay at S2 cost less to perform besides the possibility of performing more than one protein per time on the CTCs isolated by ISET® methodology. Furthermore, we previously showed that elevated TYMS expression in CTCs was associated with poor prognosis among patients with metastatic colorectal cancer [26].

TYMS, a downstream target molecule of 5-FU, plays a key role in DNA synthesis. The enzyme catalyzes deoxyuridine monophosphate methylation to produce deoxythymidine triphosphate, and subsequently, thymidylate. Increased TYMS expression is thought to be responsible for 5-FU resistance in patients with colorectal cancer [42,43]. Meanwhile, RAD23B is a member of the nucleotide excision repair system, which stabilizes the xeroderma pigmentosum complementation group C (XPC) protein and potentiates its interaction with damaged DNA [45]. XPC subsequently initiates nucleotide excision repair. It is not clear why increased production of RAD23B protein was observed in CTCs from non-responders to NCRT in the present study. A possible hypothesis is that increased levels of this repair protein during treatment make it difficult to eliminate cancer cells by NCRT, a strategy that is based on inducing apoptosis as a result of DNA damage.

A treatment approach that deserves discussion in the context of our study is the "watch and wait" strategy. For the patients whose tumors respond completely to NCRT (as evidenced by radiologic, clinical, and endoscopic evaluations), a "watch and wait" strategy may allow patients to avoid the morbidity, mortality, and functional consequences of radical surgical treatment. A recently published meta-analysis of 880 patients who were monitored with a "watch and wait" strategy showed that the strategy can be safely incorporated into a multidisciplinary management plan for the treatment of patients with rectal cancer who achieve pCR after neoadjuvant treatment [12,46–48]. Therefore, identification of a biomarker that can predict pCR to NCRT treatment prior to treatment, or in the early stages of treatment, would be of great clinical utility in combination with commonly used clinical parameters.

In the present study, it was observed that 100% of patients who responded to NCRT did not express TYMS or RAD23B proteins on their CTCs at the beginning of their follow-up monitoring. This lack of protein expression in CTCs is consistent with imaging and kinetic studies that have showed a reduction or elimination of CTCs post NCRT. Furthermore, we observed that CTC kinetics correlated with disease outcome for our patients with LARC. To the best of our knowledge, this is the first study to demonstrate this result. The observed decrease in CTC levels during treatment in our cases involving complete or partial responses further support the use of CTC analyses to predict response to NCRT.

In summary, our results provide valuable data regarding two potential biomarkers of chemoradiation resistance in patients undergoing neoadjuvant treatment for LARC. Despite the small sample size of our study, CTC kinetics, as well as *TYMS* mRNA and/or RAD23B/TYMS protein expression in CTCs, were found to strongly correlate with pCR. Further studies are needed to validate these findings with a larger patient cohort. If CTC analysis proves useful in predicting pCR with high accuracy, many patients may be spared radical surgery for rectal cancer treatment. In addition, biomarker and kinetic analyses of CTCs may identify potential non-responders to treatment with NCRT, thereby identifying a need to evaluate other forms of therapy for these patients.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/7/641/s1, Table S1: table of sensitivity, specificity and predictive values of biomarkers evaluated.

Author Contributions: B.C.T.E: conception/design, data analysis and interpretation, collection and/or assembly of data, manuscript writing; V.S.e.S.: conception/design, provision of study material or patients, collection and/or assembly of data, manuscript writing; E.A.A.: data analysis and interpretation; C.A.L.M.: provision of study material or patients; M.L.G.S.: data analysis and interpretation; G.G.M.: data analysis and interpretation; A.C.B.: data analysis and interpretation; S.A.J.: conception/design; L.T.D.C.: conception/design, data analysis and interpretation, manuscript writing, final approval of manuscript.

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References

1. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2018. *CA Cancer J. Clin.* **2018**, *68*, 7–30. [CrossRef] [PubMed]
2. Cravo, M.; Rodrigues, T.; Ouro, S.; Ferreira, A.; Féria, L.; Maio, R. Management of rectal cancer: Times they are changing. *GE Port. J. Gastroenterol.* **2014**, *21*, 192–200. [CrossRef]
3. Gaertner, W.B.; Kwaan, M.R.; Madoff, R.D.; Melton, G.B. Rectal cancer: An evidence-based update for primary care providers. *World J. Gastroenterol.* **2015**, *21*, 7659–7671. [CrossRef] [PubMed]
4. Li, Y.; Wang, J.; Ma, X.; Tan, L.; Yan, Y.; Xue, C.; Hui, B.; Liu, R.; Ma, H.; Ren, J. A Review of Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer. *Int. J. Biol. Sci.* **2016**, *12*, 1022–1031. [CrossRef] [PubMed]
5. Ryan, J.E.; Warrier, S.K.; Lynch, A.C.; Ramsay, R.G.; Phillips, W.A.; Heriot, A.G. Predicting pathological complete response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer: a systematic review. *Colorectal Dis.* **2016**, *18*, 234–246. [CrossRef] [PubMed]
6. Zou, X.-C.; Wang, Q.-W.; Zhang, J.-M. Comparison of 5-FU-based and Capecitabine-based Neoadjuvant Chemoradiotherapy in Patients With Rectal Cancer: A Meta-analysis. *Clin. Colorectal Cancer* **2017**, *16*, e123–e139. [CrossRef] [PubMed]
7. Schmoll, H.-J.; Haustermans, K.; Price, T.J.; Nordlinger, B.; Hofheinz, R.; Daisne, J.-F.; Janssens, J.; Brenner, B.; Schmidt, P.; Reinel, H.; et al. Preoperative chemoradiotherapy and postoperative chemotherapy with capecitabine +/- oxaliplatin in locally advanced rectal cancer: Final results of PETACC-6. *JCO* **2018**, *36*, 3500. [CrossRef]
8. Porzo, M.E.; Fang, S.H. Watch and wait approach to rectal cancer: A review. *World J. Gastrointest. Surg.* **2015**, *7*, 306–312. [CrossRef]
9. Vojtíšek, R.; Kořtáková, E.; Mařan, J.; Šonejs, O.; Finek, J. Neoadjuvant chemoradiotherapy of the rectal carcinoma - The correlation between the findings on the restaging multiparametric 3T MRI scanning and the surgical findings. *Rep. Pract. Oncol. Radiother.* **2017**, *22*, 265–276. [CrossRef]
10. Molinari, C.; Matteucci, E.; Caroli, P.; Passardi, A. Biomarkers and Molecular Imaging as Predictors of Response to Neoadjuvant Chemoradiotherapy in Patients With Locally Advanced Rectal Cancer. *Clin. Colorectal Cancer* **2015**, *14*, 227–238. [CrossRef]
11. van der Valk, M.J.M.; Hilling, D.E.; Bastiaannet, E.; Meershoek-Klein Kranenbarg, E.; Beets, G.L.; Figueiredo, N.L.; Habr-Gama, A.; Perez, R.O.; Renehan, A.G.; van de Velde, C.J.H.; et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. *Lancet* **2018**, *391*, 2537–2545. [PubMed]
12. Fernandez-Martos, C.; Garcia-Albeniz, X.; Pericay, C.; Maurel, J.; Aparicio, J.; Montagut, C.; Safont, M.J.; Salud, A.; Vera, R.; Massuti, B.; et al. Chemoradiation, surgery and adjuvant chemotherapy versus induction chemotherapy followed by chemoradiation and surgery: long-term results of the Spanish GCR-3 phase II randomized trial†. *Ann. Oncol.* **2015**, *26*, 1722–1728. [CrossRef] [PubMed]
13. Cercek, A.; Roxburgh, C.S.D.; Strombom, P.; Smith, J.J.; Temple, L.K.F.; Nash, G.M.; Guillem, J.G.; Paty, P.B.; Yaeger, R.; Stadler, Z.K.; et al. Adoption of Total Neoadjuvant Therapy for Locally Advanced Rectal Cancer. *JAMA Oncol.* **2018**, *4*, e180071. [CrossRef] [PubMed]
14. Yamashita, K.; Matsuda, T.; Hasegawa, H.; Mukohyama, J.; Arimoto, A.; Tanaka, T.; Yamamoto, M.; Matsuda, Y.; Kanaji, S.; Nakamura, T.; et al. Recent advances of neoadjuvant chemoradiotherapy in rectal cancer: Future treatment perspectives. *Ann. Gastroenterol. Surg.* **2019**, *3*, 24–33. [CrossRef] [PubMed]

15. Sun, W.; Li, G.; Wan, J.; Zhu, J.; Shen, W.; Zhang, Z. Circulating tumor cells: A promising marker of predicting tumor response in rectal cancer patients receiving neoadjuvant chemo-radiation therapy. *Oncotarget* **2016**, *7*, 69507–69517. [CrossRef] [PubMed]
16. Cristofanilli, M.; Hayes, D.F.; Budd, G.T.; Ellis, M.J.; Stopeck, A.; Reuben, J.M.; Doyle, G.V.; Matera, J.; Allard, W.J.; Miller, M.C.; et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J. Clin. Oncol.* **2005**, *23*, 1420–1430. [CrossRef]
17. Cohen, S.J.; Punt, C.J.A.; Iannotti, N.; Saidman, B.H.; Sabbath, K.D.; Gabrail, N.Y.; Picus, J.; Morse, M.; Mitchell, E.; Miller, M.C.; et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **2008**, *26*, 3213–3221. [CrossRef]
18. León-Mateos, L.; Viteito, M.; Anido, U.; López López, R.; Muinelo Romay, L. Clinical Application of Circulating Tumour Cells in Prostate Cancer: From Bench to Bedside and Back. *Int. J. Mol. Sci.* **2016**, *17*, 1580. [CrossRef]
19. Souza E Silva, V.; Chinen, L.T.D.; Abdallah, E.A.; Damascena, A.; Paludo, J.; Chojniak, R.; Dettino, A.L.A.; de Mello, C.A.L.; Alves, V.S.; Fanelli, M.F. Early detection of poor outcome in patients with metastatic colorectal cancer: tumor kinetics evaluated by circulating tumor cells. *Onco. Targets Ther.* **2016**, *9*, 7503–7513. [CrossRef]
20. Yan, W.-T.; Cui, X.; Chen, Q.; Li, Y.-E; Cui, Y.-H.; Wang, Y.; Jiang, J. Circulating tumor cell status monitors the treatment responses in breast cancer patients: a meta-analysis. *Sci Rep* **2017**, *7*, 43464. [CrossRef]
21. Benini, S.; Gamberi, G.; Cocchi, S.; Garbetta, J.; Alberti, L.; Righi, A.; Gambarotti, M.; Picci, P.; Ferrari, S. Detection of circulating tumor cells in liquid biopsy from Ewing sarcoma patients. *Cancer Manag. Res.* **2018**, *10*, 49–60. [CrossRef] [PubMed]
22. Pantel, K.; Alix-Panabières, C. Liquid biopsy and minimal residual disease–latest advances and implications for cure. *Nat. Rev. Clin. Oncol.* **2019**. [CrossRef]
23. Kuremsky, J.G.; Tepper, J.E.; McLeod, H.L. Biomarkers for response to neoadjuvant chemoradiation for rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **2009**, *74*, 673–688. [CrossRef] [PubMed]
24. Conradi, L.-C.; Bleckmann, A.; Schirmer, M.; Spaenger, T.; Jo, P.; Homayounfar, K.; Wolff, H.A.; Rothe, H.; Middel, P.; Becker, H.; et al. Thymidylate synthase as a prognostic biomarker for locally advanced rectal cancer after multimodal treatment. *Ann. Surg. Oncol.* **2011**, *18*, 2442–2452. [CrossRef] [PubMed]
25. Watkins, J.F.; Sung, P.; Prakash, L.; Prakash, S. The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Mol. Cell. Biol.* **1993**, *13*, 7757–7765. [CrossRef] [PubMed]
26. Schauber, C.; Chen, L.; Tongaonkar, P.; Vega, I.; Lambertson, D.; Potts, W.; Madura, K. Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* **1998**, *391*, 715–718. [CrossRef] [PubMed]
27. Pérez-Mayoral, J.; Pacheco-Torres, A.L.; Morales, L.; Acosta-Rodríguez, H.; Matta, J.L.; Dutil, J. Genetic polymorphisms in RAD23B and XPC modulate DNA repair capacity and breast cancer risk in Puerto Rican women. *Mol. Carcinog.* **2013**, *52*, E127–E138. [CrossRef]
28. Abdallah, E.A.; Fanelli, M.F.; Buim, M.E.C.; Machado Netto, M.C.; Gasparini Junior, J.L.; Souza E Silva, V.; Dettino, A.L.A.; Mingues, N.B.; Romero, J.V.; Ocea, L.M.M.; et al. Thymidylate synthase expression in circulating tumor cells: a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. *Int. J. Cancer* **2015**, *137*, 1397–1405. [CrossRef]
29. Krebs, M.G.; Hou, J.-M.; Sloane, R.; Lancashire, L.; Priest, L.; Nonaka, D.; Ward, T.H.; Backen, A.; Clack, G.; Hughes, A.; et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. *J. Thorac. Oncol.* **2012**, *7*, 306–315. [CrossRef]
30. Khoja, L.; Backen, A.; Sloane, R.; Menasce, L.; Ryder, D.; Krebs, M.; Board, R.; Clack, G.; Hughes, A.; Blackhall, F.; et al. A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. *Br. J. Cancer* **2012**, *106*, 508–516. [CrossRef]
31. Sung, S.; Son, S.H.; Park, E.Y.; Kay, C.S. Prognosis of locally advanced rectal cancer can be predicted more accurately using pre- and post-chemoradiotherapy neutrophil-lymphocyte ratios in patients who received preoperative chemoradiotherapy. *PLoS ONE* **2017**, *12*, e0173955. [CrossRef] [PubMed]
32. Powell, A.A.; Talasaz, A.H.; Zhang, H.; Coram, M.A.; Reddy, A.; Deng, G.; Telli, M.L.; Advani, R.H.; Carlson, R.W.; Mollick, J.A.; et al. Single Cell Profiling of Circulating Tumor Cells: Transcriptional Heterogeneity and Diversity from Breast Cancer Cell Lines. *PLoS ONE* **2012**, *7*, e33788. [CrossRef] [PubMed]

33. Gavenbaum, A.; Martin, D.R.; Bocklage, T.; Lee, J.-H.; Ness, S.A.; Rajput, A. Tumor Heterogeneity as a Predictor of Response to Neoadjuvant Chemotherapy in Locally Advanced Rectal Cancer. *Clin. Colorectal Cancer* **2019**. [CrossRef] [PubMed]
34. Franke, A.J.; Parekh, H.; Starr, J.S.; Tan, S.A.; Iqbal, A.; George, T.J. Total Neoadjuvant Therapy: A Shifting Paradigm in Locally Advanced Rectal Cancer Management. *Clin. Colorectal Cancer* **2018**, *17*, 1–12. [CrossRef] [PubMed]
35. Garcia-Aguilar, J.; Chen, Z.; Smith, D.D.; Li, W.; Madoff, R.D.; Cataldo, P.; Marcet, J.; Pastor, C. Identification of a biomarker profile associated with resistance to neoadjuvant chemoradiation therapy in rectal cancer. *Ann. Surg.* **2011**, *254*, 486–492, discussion 492-493. [CrossRef]
36. Santos, M.D.; Silva, C.; Rocha, A.; Nogueira, C.; Castro-Poças, F.; Araujo, A.; Matos, E.; Pereira, C.; Medeiros, R.; Lopes, C. Predictive clinical model of tumor response after chemoradiation in rectal cancer. *Oncotarget* **2017**, *8*, 58133–58151. [CrossRef]
37. Sun, Y.; Wu, X.; Zhang, Y.; Lin, H.; Lu, X.; Huang, Y.; Chi, P. Pathological complete response may underestimate distant metastasis in locally advanced rectal cancer following neoadjuvant chemoradiotherapy and radical surgery: Incidence, metastatic pattern, and risk factors. *Eur. J. Surg. Oncol.* **2019**. [CrossRef]
38. Gotanda, K.; Hirota, T.; Matsumoto, N.; Ieiri, I. MicroRNA-433 negatively regulates the expression of thymidylate synthase (TYMS) responsible for 5-fluorouracil sensitivity in HeLa cells. *BMC Cancer* **2013**, *13*, 369. [CrossRef]
39. Kwon, M.J.; Soh, J.S.; Lim, S.-W.; Kang, H.S.; Lim, H. HER2 as a limited predictor of the therapeutic response to neoadjuvant therapy in locally advanced rectal cancer. *Pathol.–Res. Pract.* **2019**, *215*, 910–917. [CrossRef]
40. Kaneno, R.; Shurin, G.V.; Kaneno, F.M.; Naiditch, H.; Luo, J.; Shurin, M.R. Chemotherapeutic agents in low noncytotoxic concentrations increase immunogenicity of human colon cancer cells. *Cell Oncol. (Dordr)* **2011**, *34*, 97–106. [CrossRef]
41. Lee, S.-C.; Xu, X.; Lim, Y.-W.; Iau, P.; Sukri, N.; Lim, S.-E.; Yap, H.L.; Yeo, W.-L.; Tan, P.; Tan, S.-H.; et al. Chemotherapy-induced tumor gene expression changes in human breast cancers. *Pharmacogenet. Genomics* **2009**, *19*, 181–192. [CrossRef] [PubMed]
42. Negrei, C.; Hudita, A.; Ginghina, O.; Galateanu, B.; Voicu, S.N.; Stan, M.; Costache, M.; Fenga, C.; Drakoulis, N.; Tsatsakis, A.M. Colon Cancer Cells Gene Expression Signature As Response to 5- Fluorouracil, Oxaliplatin, and Folinic Acid Treatment. *Front. Pharmacol.* **2016**, *7*, 172. [CrossRef] [PubMed]
43. Tang, M.; Lu, X.; Zhang, C.; Du, C.; Cao, L.; Hou, T.; Li, Z.; Tu, B.; Cao, Z.; Li, Y.; et al. Downregulation of SIRT7 by 5-fluorouracil induces radiosensitivity in human colorectal cancer. *Theranostics* **2017**, *7*, 1346–1359. [CrossRef]
44. Liang, X.; Shi, H.; Yang, L.; Qiu, C.; Lin, S.; Qi, Y.; Li, J.; Zhao, A.; Liu, J. Inhibition of polypyrimidine tract-binding protein 3 induces apoptosis and cell cycle arrest, and enhances the cytotoxicity of 5- fluorouracil in gastric cancer cells. *Br. J. Cancer* **2017**, *116*, 903–911. [CrossRef] [PubMed]
45. Ng, J.M.Y.; Vermeulen, W.; van der Horst, G.T.J.; Bergink, S.; Sugasawa, K.; Vrieling, H.; Hoeijmakers, J.H.J. A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. *Genes Dev.* **2003**, *17*, 1630–1645. [CrossRef] [PubMed]
46. Xu, W.; Jiang, H.; Zhang, F.; Gao, J.; Hou, J. MicroRNA-330 inhibited cell proliferation and enhanced chemosensitivity to 5-fluorouracil in colorectal cancer by directly targeting thymidylate synthase. *Oncol. Lett.* **2017**, *13*, 3387–3394. [CrossRef]
47. Bunick, C.G.; Miller, M.R.; Fuller, B.E.; Fanning, E.; Chazin, W.J. Biochemical and structural domain analysis of xeroderma pigmentosum complementation group C protein. *Biochemistry* **2006**, *45*, 14965–14979. [CrossRef] [PubMed]
48. Rushworth, D.; Mathews, A.; Alpert, A.; Cooper, L.J.N. Dihydrofolate Reductase and Thymidylate Synthase Transgenes Resistant to Methotrexate Interact to Permit Novel Transgene Regulation. *J. Biol. Chem.* **2015**, *290*, 22970–22976. [CrossRef]

Anexo 4 - Artigo 2 publicado

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Article

Baseline and Kinetic Circulating Tumor Cell Counts Are Prognostic Factors in a Prospective Study of Metastatic Colorectal Cancer

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Abstract: The discovery of predictive biomarkers in metastatic colorectal cancer (mCRC) is essential to improve clinical outcomes. Recent data suggest a potential role of circulating tumor cells (CTCs) as prognostic indicators. We conducted a follow-on analysis from a prospective study of consecutive patients with mCRC. CTC analysis was conducted at two timepoints: baseline (CTC1; before starting chemotherapy), and two months after starting treatment (CTC2). CTC isolation/quantification were completed by ISET® (Rarecells, France). CTC expressions of drug resistance-associated proteins were evaluated. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan–Meier method. Seventy-five patients were enrolled from May 2012 to May 2014. A CTC1 cut-off of >1.5 CTCs/mL was associated with an inferior median OS compared to lower values. A difference of CTC2—CTC1 > 5.5 CTCs/mL was associated with a reduced median PFS. By multivariate analysis, CTC1 > 1.5 CTCs/mL was an independent prognostic factor for worse OS. Multi-drug resistance protein-1 (MRP-1) expression was associated with poor median OS. CTC baseline counts, kinetics, and MRP-1 expression were predictive of clinical outcomes. Larger studies are warranted to explore the potential clinical benefit of treating mCRC patients with targeted therapeutic regimens guided by CTC findings.

Keywords: circulating tumor cells; kinetics; metastatic colorectal cancer; prognosis; multidrug resistance protein 1 (MRP-1)

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1. Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal tumors, constituting the second most common cancer diagnosed in women and third most common in men, and accounting for approximately 10% of all incident cancers and cancer-related deaths worldwide [1]. Approximately 50% to 60% of CRC patients develop metastases, with 80% to 90% of these patients afflicted with unresectable hepatic lesions [2–4].

The management of metastatic colorectal cancer (mCRC) has advanced significantly in recent years [5]. Treatments have evolved from the time when 5-fluorouracil (5-FU) was the only active agent and conferred an overall survival (OS) of approximately 11 to 12 months, to current therapies that result in an average OS that is reaching three years. These improvements have been driven primarily by the availability of new active agents that include cytotoxic drugs such as oxaliplatin and irinotecan, and monoclonal antibodies

that inhibit angiogenesis and proliferation pathways by targeting vascular endothelial growth factor and the epidermal growth factor receptor, respectively [5–9].

Despite these advances, the heterogeneity of mCRC impedes efforts by the oncologist to design targeted treatment strategies and to optimize personalized therapy. Consequently, the development of predictive tools that can be applied to each patient before the initiation of treatment becomes essential for clinical practice [10–12]. In addition, the discovery of circulating biomarkers to improve patient management is imperative due to the limitations of the currently available methods of tissue biopsy and radiological evaluation for follow-up and prognosis. Thus, in the era of precision medicine, the liquid biopsy has become an important decision-support tool for the oncologist in the design of treatment strategies [13–17].

Liquid biopsy constitutes real time analysis of tumor and/or metastasis components by body fluids, such as blood, urine, feces, saliva and allows the assessment of the complexity and heterogeneity of the disease. Liquid biopsy components are circulating tumor cells, circulating tumor DNA and microRNAs, tumor-derived extracellular vesicles and thus, provides a less invasive and dynamic way to predict a recurrence and resistance to the proposed treatment [18]. Circulating tumor cells (CTCs) are released by primary and/or metastatic cancer during their formation and/or tumor progression [19]. Numerous previous studies have shown that CTC counts can predict progression-free survival (PFS) and OS in patients with early and metastatic breast cancer, metastatic prostate cancer and CRC [20–23]. There are currently 963 clinical trials using CTCs worldwide (clinicaltrials.gov, accessed on the 9 March 2021), representing the interest in the applicability of liquid biopsy in the management of oncology patients [24].

Therefore, there are perspectives for CTCs to be used in the CRC for screening (early detection of invasive cancers); in localized cancer (risk stratification, prognosis and monitoring after treatment); and in metastatic disease (treatment design, response monitoring and identification of drug resistance mechanisms). However, clinical validation is still necessary [25–27]. In addition, as there are many different methods to isolate CTCs, it is also fundamental to standardize the methodology and many international efforts have been made in this sense. The majority of the technologies described in the literature use EpCAM to isolate these cells, however, there are controversies about the use of this molecule to isolate CTCs from colorectal tumor origins. In the review paper from Eslami-S et al. [28], the authors comment about the variation of EpCAM positive CTCs among different types of solid cancers, that high numbers of EpCAM positive CTCs are often detected in blood samples from patients with breast, prostate and small cell lung cancer. Those counts (EpCAM + CTC) are low in patients with pancreatic, colorectal and non-small cell lung cancer. In metastatic colorectal cancer, per example, CTC counts vary greatly between studies, with around 10% to 30% of CTC-positive patients at baseline before treatment. Our group have been working with ISET (Isolation by SizE of Tumors, Rarecells, France) for a long time and have published studies in colorectal cancer, with higher detection rates, around 88.5% [29,30]. It can be explained by the possibility to isolate cells independently of the marker, so, CTCs under epithelial–mesenchymal transition can be easily taken.

Here, we have a follow-on study, with the objective to evaluate the real impact of CTCs in OS and PFS. With part of the cohort included, we previously demonstrated the importance of kinetic evaluations of CTCs [26] and assessments of CTC expressions of thymidylate synthase (TYMS) and multidrug resistance protein-1 (MRP-1) as potential predictive biomarkers in mCRC patients [29,31]. So, we analyzed data set published previously by our group (with 54 patients) combined with an additional sample (more 21 patients) to determine if CTC (counts and kinetics and biomarkers) would remain as prognostic indicators.

2. Materials and Methods

2.1. Patient Recruitment

This follow-on analysis of a prospective longitudinal study was conducted at the A.C. Camargo Cancer Center (Rua Professor Antônio Prudente, 211, Liberdade; São Paulo, Brazil) and was approved by the local Ethics Committee (CEP 1367/10B-10 April 2012). Written informed consent was obtained from all patients prior to enrollment. Inclusion criteria included a diagnosis of inoperable metastatic colorectal cancer, confirmed by histopathology of the primary and/or metastatic lesion and radiological analysis; age of ≥18 years; Eastern Clinical Oncology Group Performance Status (ECOG PS) of 0–2 without organ dysfunction; and an intent to begin chemotherapy for metastatic disease. Cancer stage was determined by the results of a physical examination and diagnostic imaging, and was scored by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria v.1.1 [32] Patients were designated by numerical code to preserve confidentiality.

2.2. Study Design

We conducted a prospective cohort study. All participants had 8 mL of blood collected at two different timepoints: baseline (CTC1; before starting treatment with either first, second, or third-line chemotherapy; after the diagnosis of metastasis or tumor progression; or at the initiation of a new chemotherapy regimen); and two months after the start of treatment (CTC2), when radiological imaging was performed.

Patients underwent computed tomography and/or magnetic resonance imaging of the thorax, abdomen, and pelvis before CTC1 collection and approximately every 2–4 months during treatment, or as clinically indicated in the judgment of the attending physician. Images were interpreted using the RECIST criteria v.1.1. The clinicopathological information of the patients and the mutational status of *RAS* in the tumors were collected from medical records.

2.3. CTC Isolation and Identification

Blood (8.0 mL) was collected in ethylenediaminetetraacetic acid (EDTA) tubes and maintained under homogenization until processing (approximately 4 h) by the Isolation by SizE of Tumor cell technique (ISET®; Rarecells, France) according to the manufacturer's instructions. To identify and analyze CTCs, we performed immunocytochemistry (ICC) using the following antibodies: anti-thymidilate synthase (TYMS) (WH0007298M1; Sigma-Aldrich, San Luis, MO, USA) to verify 5-FU resistance; anti-excision repair cross-complementation group 1 protein (ERCC1) (SAB4500795; Sigma-Aldrich, San Luis, MO, USA) to verify oxaliplatin resistance; and anti-multidrug resistance-associated protein-1 (MRP-1) (HPA002380; Sigma-Aldrich, San Luis, MO, USA) to determine irinotecan resistance. We also performed ICC against the leukocyte common antigen CD45 (CSB-PA010546; CusaBio, Houston, TX, USA) to exclude leucocytes from our analysis. After the ICC assay (performed as described by Abdallah et al. 2016 [29]), spots on ISET® membranes were counterstained with hematoxylin and analyzed by light microscopy. We conducted cytopathologic analysis of CTCs according to the following parameters: high nuclear–cytoplasmic ratio (>0.8), cell diameter >16 µm; hyperchromatic and irregular nuclei [33]; and negative CD45 reactivity.

2.4. CTC Count

We considered the CTC count as a continuous variable and calculated cut-off points to discriminate between two groups of patients, those with good versus those with poor clinical outcomes. The cut-offs for each event of interest (OS and PFS) were estimated as previously reported [34].

2.5. Statistical Analysis

Baseline patient characteristics were expressed as absolute and relative frequencies for qualitative variables and as the median, minimum and maximum for quantitative variables.

Regarding CTC counts at the two timepoints, the determination of two groups of observations by a simple cut-off point was estimated using the maximum of the standardized log-rank statistic [34]. Simple cut-off points to differentiate groups of observations CTC counts at each timepoint were estimated by using the maximum of the standardized log-rank statistic. In other words, CTC1 and delta CTC (CTC2-CTC1) values were calculated by Lausen and Schumacher method [34], which aims to determine the best cut-off point in order to discriminate the survival functions. The value 1.5 for CTC1 and 5.5 for delta CTC obtained were placed as cut-offs, as qualitative variables (Supplementary Figures S1 and S2). Then, we estimated the survival curves using the Kaplan–Meier estimator and the covariate's effect was evaluated by means of Cox regression proportional hazards model.

Survival functions were estimated by the Kaplan–Meier method, and curves were compared by using the log-rank test. The Cox semi-parametric proportional hazards model was fitted to evaluate relationships between covariates and overall survival/progression-free survival [35]. The assumption of proportional hazards was assessed on the so-called Schoenfeld residuals [36,37]. There was evidence that covariates had a constant effect over time in all cases. Overall survival was calculated from the date of CTC1 collection to the date of first recurrence, determined by diagnostic imaging or last follow-up. The significance level was fixed at 5% for all tests. Statistical analyses were performed using IBM SPSS Statistics version 23.0 (IBM Corp., Armonk, NY, USA) and R software version 3.5 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Patient Characteristics

Seventy-five consecutive mCRC patients were enrolled from May 2012 to May 2014. Mean age was 59 years (24–81 years), and most were male (n = 42; 56%). All had metastatic disease at the time of enrollment, including 22.7% (n = 17) with metastasis confined to the liver, and 37.3% (n = 28) with extra-hepatic and hepatic metastasis. Most patients had an ECOG PS of 0 or 1, and none had contraindications to systemic chemotherapy. Forty-one percent (n = 31) had undergone previous surgical resections of metastases; 25% (n = 19), 12% (n = 9), and 4% (n = 3) had resections of hepatic, pulmonary, and hepatic and pulmonary metastases, respectively (Table 1). Baseline CTC1 analysis was completed before the initiation of first-line chemotherapy in 38/75 patients (50.6%); before the initiation of second-line therapy (after disease progression during first-line therapy) in 20/75 patients (26.7%); and after progression during second-line therapy in 17/75 patients (22.7%).

Table 1. Clinicopathological features.

Variable	No	%
Total number of patients	75	100
Age (in years) Median (Min-Max); Mean (SD)	59 (24–81)	57.32 (12.87)
Gender		
Male	42	56
Female	33	44
ECOG OS		
0	43	60
1	25	33
2	7	7
Laterality		
Left side	54	72
Right side	19	25.3
Unknown	2	2.7

Table 1. *Cont.*

Variable	No	%
Location of metastasis		
Only hepatic	17	22.7
Hepatic and extra-hepatic	28	37.3
Other (pulmonary, lymph node and or peritoneal)	30	40
First-line treatment		
Oxaliplatin-based	41	54.6
Irinotecan-based	32	42.7
No chemotherapy	2	2.7
Line of treatment (at CTC1 collection)		
First-line	38	50.6
Second-line	20	26.7
Third-line	17	22.7
Metastasis resection		
Hepatic	19	25
Pulmonary	9	12
Hepatic and Pulmonary	3	4
No resection	44	59
RAS status		
Wild-type	41	54.7
Mutant *	33	44
Unknown	1	1.3

ECOG PS: Eastern Cooperative Oncology Group Performance Status; SD: standard deviation. * Related to the 33 patients with *RAS* mutations, 21 had *KRAS* codon 12 mutations, nine had *KRAS* codon 13 mutations, two had *KRAS* codon 146 mutations, and one had an *NRAS* codon 12 mutation.

Median survival was 34.5 months (95% confidence interval (CI), 28.3–40.7 months). *RAS* mutants were associated with a median survival of 20.1 months (95% CI, 10.7–29.5 months), which was significantly shorter than that of patients with wild-type *RAS*, in whom median survival was 41.0 months (95% CI, 32.3–49.7 months); log-rank $p = 0.001$). Patients with right-sided colon tumors had an inferior median survival (20.5 months, 95% CI, 11.3–29.9) when compared with patients with left-sided cancers (39.5, 95% CI, 30.3–48.7 months) (log-rank $p = 0.015$).

3.2. *Prognostic Value of CTCs*

The best estimated cut-off point for CTC1 and OS for the entire cohort was 1.5 CTCs/mL (median 2.5 CTCs/mL, range 0–31.2 CTCs/mL). Patients with CTC1 > 1.5 had a median OS of 24.5 months (95% CI, 9.5–39.4 months), less than the OS of patients with CTC $\leq$ 1.5 (34.2 months (95% CI, 18.4–50.2 months); log-rank $p = 0.041$) (Figure 1).

Patients with CTC1 > 1.5 had a higher risk of death than patients with CTC1 $\leq$ 1.5 CTCs/mL (hazard ratio (HR) = 1.893; 95% CI, 1.015–3.528; $p = 0.041$) (Table 2). Patients with CTC2—CTC1 > 5.5 per mL demonstrated poorer median progression-free survival (PFS) (3.2 months; 95% CI, 0.001–6.5 months) when compared to CTC2—CTC1 $\leq$ 5.5 (9.1 months; 95% CI, 7.1–11.1 months; log-rank $p = 0.005$). Patients with CTC2–CTC1 > 5.5 had a higher risk of death than those with CTC2–CTC1 $\leq$ 5.5 CTCs/mL (HR = 3.107; 95% CI, 1.34–7.22; $p = 0.01$) (Table 3).

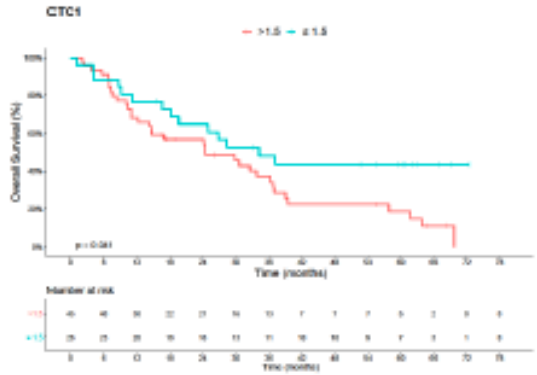

Figure 1. Kaplan–Meier curves of overall survival in the general studied population (75 patients with metastatic colorectal cancer). In blue, survival curve for patient with ≤1.5 CTC/mL. In red, patients with >1.5 CTC/mL ($p = 0.041$).

Table 2. Estimate of the parameters of the simple and multiple Cox regression model for overall survival from metastatic colon cancer.

Variable	Category	Simple Cox Regression Model			Multiple Cox Regression Model		
		HR	95% CI	*p*	HR	95% CI	*p*
CTC1	≤1.5	Ref			Ref		
	>1.5	1.893	1.015–3.528	0.041	2.34	1.113–4.919	0.025
MRP1	Negative						
	Positive	4.791	1.051–21.829	0.043			
***RAS* status**	Wild	Ref			Ref		
	Mutated	3.306	1.672–6.531	0.001	5.467	2.320–12.886	<0.0001
Laterality	Left side	Ref			Ref		
	Right side	2.246	1.178–4.282	0.014	2.166	0.984–4.767	0.055

HR: hazard ratio; Ref: reference category.

Table 3. Estimate of the parameters of the simple and multiple Cox regression model for progression-free survival from metastatic colon cancer.

Variable	Category	Simple Cox Model			Multiple Cox Model		
		HR	95% CI	*p*	HR	95% CI	*p*
CTC2−CTC1	≤5.5	Ref			Ref		
	>5.5	3.107	1.340–7.220	0.010	2.373	0.950–5.918	0.064
MRP1	Negative	Ref					
	Positive	6.933	1.689–28.454	0.007			
***RAS* status**	Wild	Ref			Ref		
	Mutated	3.044	1.602–5.783	0.001	8.362	2.719–25.717	<0.0001
Laterality	Left side	Ref			Ref		
	Right side	1.659	0.929–2.902	0.087	0.826	0.339–2.009	0.673

HR: hazard ratio; Ref: reference category.

Among the 38 patients receiving first-line therapy, those with CTC1 > 1.0 had a median OS of 29.74 months (95% CI, 17.9–41.6); in patients with CTC1 ≤ 1.0 median OS was not reached (log rank $p = 0.09$; HR = 2.5, 95% CI, 0.83–7.53, $p = 0.103$). We estimated a cut-off of CTC2−CTC1 > 2.0/mL for PFS in this group. The median PFS was 4.5 months (95%

CI, 1.7–7.3 months) for patients with CTC2−CTC1 > 2.0/mL, and 22.3 months (95% CI, 8.0–36.5 months) for those with CTC2−CTC1 ≤ 2.0/mL (log rank $p = 0.001$).

For the 20 patients undergoing second-line treatment, the estimated CTC1 cut-off was 1.65 CTCs/mL for OS. Median OS values were 29.0 months (95% CI, 29.9–58.0) for patients with CTC1 > 1.65/mL, and 43.5 months (95% CI, 9.2–48.9 months) for those with CTC1 ≤ 1.65/mL (log rank $p = 0.29$). In this group, the estimated cut-off for PFS was CTC2−CTC1 was 1.0 CTCs/mL; patients with CTC2−CTC1 ≤ 1.0 showed a median PFS of 14.0 months (95% CI, 2.3–25.7 months) versus 8.8 months (95% CI, 5.1–12.6 months) for those with >1.0/mL (log rank $p = 0.36$).

3.3. CTC Biomarkers

Evaluation of CTC MRP-1 expression was limited to 19 patients due to restricted availability of materials (here, we evaluated the same 19 patients included in the paper published by Abdallah et al. [29] and analyzed the power of the variable MRP-1 with the extended follow-up). MRP-1 expression was associated with worse median OS (4.21 months, 95% CI: 0.001–12.4 months) compared to negative MRP-1 expression (32.6 months, 95% CI, 19.2–46 months; log rank $p = 0.026$). Despite the limited sample size, we demonstrated that MRP-1 expression in CTCs increased the risks of death (HR = 4.791, 95% CI, 1.05–21.83; $p = 0.043$) and disease progression (HR = 6.933, 95% CI, 1.69–28.45; $p = 0.007$) (Tables 2 and 3).

ERCC1 expression was evaluated in 13 patients (data previously published by Abdallah et al. [29] with updated follow-up). There was a trend towards shorter OS in ERCC1-positive compared to ERCC1-negative patients that did not reach statistical significance (median OS of 29.7 months (95% CI, 10.9–48.5 vs. 36.8, 95% CI, 0.001–102 months; log-rank $p = 0.38$). TYMS expression was analyzed in 36 patients (data previously published by Abdallah et al. [31] with updated follow-up). TYMS-positive patients experienced a median OS of 16.97 months (95% CI, 10.2–23.7) versus 26.8 months (95% CI, 20.6–33.1 months) for TYMS-negative patients (log-rank $p = 0.55$).

3.4. Multivariate Analysis

To evaluate the effects of independent variables on OS and PFS, we fitted a multiple Cox regression model for each outcome considering CTC1, *RAS* status, and laterality (right- versus left-sided colon as site of primary tumor) for the OS outcome; and CTC2–CTC1, *RAS* status, and laterality for the PFS outcome. Patients with CTC1 > 1.5 had a higher risk of death than those with CTC1 ≤ 1.5 CTCs/mL (HR = 2.34; 95% CI, 1.113–4.919; $p = 0.025$), and were also more likely to harbor *RAS* mutations (HR = 5.467; 95% CI, 2.32–12.886; $p = 0.001$) (Tables 2 and 3).

Multivariate analysis disclosed that *RAS* mutations were associated with shorter OS and PFS. Right-sided primary tumors were associated with a trend toward decreased OS and PFS. Unfortunately, MRP-1 expression was not analyzed in the multiple regression model due to the limited sample size (Tables 2 and 3).

4. Discussion

Due to the heterogeneity of mCRC, we have not yet discovered a sensitive and specific prognostic biomarker that can be applied in all cases to design optimal personalized treatment strategies [38,39]. We currently devise therapies for mCRC patients based on symptoms, primary tumor site, previous treatments, cancer stage, molecular evaluation (*BRAF*, *KRAS* or *NRAS* mutations and microsatellite instability), comorbidities, and treatment goals [5,40–42]. However, improvement of this initial assessment process is crucial to enhance clinical outcomes. Advances provided by liquid biopsy open new opportunities for the discovery of biomarkers to forecast more accurate prognoses, to assess drug resistance before and during therapy, and to monitor treatment response [11,25,43]. Consequently, the study of CTCs in mCRC is essential, as it allows quantitative and kinetic evaluations, as well as the identification of drug resistance-associated proteins, thus en-

abling patient-centric prognosis and evaluations of therapeutic efficacy [13,26,44,45]. CTC analysis provides a real-time image of various tumor characteristics, including the extent of heterogeneity at specific timepoints.

Numerous studies have demonstrated that baseline CTC quantification has prognostic relevance. Herein, our study of 75 mCRC patients receiving different lines of chemotherapy and varied treatment regimens showed that CTC1 > 1.5/mL was an independent prognostic indicator of OS. In addition, CTC1 > 1.5 CTCs/mL was associated with decreased OS in patients receiving first-line therapy. We suggest that CTC quantification before the start of chemotherapy, combined with other clinical criteria and molecular diagnostics, can predict clinical outcomes and thereby assist the physician in designing treatment.

In an earlier study of 54 patients, we demonstrated that CTC kinetics were important prognostic indicators [26]. Our current study associated higher increases in CTC counts with reduced PFS in the entire cohort and in first-line chemotherapy recipients (CTC2—CTC1 cut-offs of >5.5 per mL and >2.0/mL, respectively). These results are concordant with our previous findings and demonstrate the clinical relevance of CTC kinetics.

In addition to conducting quantitative and kinetic analyses of CTCs, we assessed drug resistance-associated protein expressions in CTCs that may predict treatment response and enable the selection of personalized treatment regimens. Consequently, this study evaluated proteins investigated by our group in previous publications [29,31]. CTC MRP-1 expression was related to reduced PFS in an earlier study by our group [29]. In the current analysis, we confirmed that in addition to the previously demonstrated relationship with worse PFS, MPR-1 expression was associated with a major reduction in OS. Yang et al. [46] evaluated 116 patients with colon adenocarcinoma, and related MPR-1 expression in tumor tissue to poor prognosis. Their results, together with ours, highlight the clinical relevance of CTC MRP-1 expression as a prognostic biomarker and predictor of therapeutic response that can be used in future clinical studies and, if possible, in clinical practice to select MRP-1-positive patients for more intensive treatments.

5. Conclusions

In conclusion, our study of a heterogenous sample of 75 mCRC patients demonstrated the clinical utility of CTC assessments. Quantitative, kinetic, and resistance protein analyses of CTCs, together with other clinical and molecular factors, allow improved individualized prognostic forecasts. Our study suggests that CTCs represent a promising tool for future clinical investigations to corroborate these findings, and thus enable the oncologist to select patients whose cancers are more likely to respond to particular treatment regimens, and to optimize the sequence of therapeutic options for patients with mCRC.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4418/11/3/502/s1, Figure S1: Lausen and Schumacher method to determine the best cut-off point for CTC1, Figure S2: Lausen and Schumacher method to determine the best cut-off point for delta CTC (CTC2-CTC1).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of A.C Camargo Cancer Center (São Paulo, Brazil) and was approved by the local Ethics Committee (CEP 1367/10B).

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

Data Availability Statement: Not applicable. The data presented in this study are available on request from the corresponding author. The data are not publicly available because they are organized in a bank data that does not allow public sharing.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]
2. Chan, K.; Wu, T.; Wang, Y.; Lee, C.; Wu, T.; Chou, H.; Lee, W.; Chiang, J.; Chen, J. Clinical relevance of oncologic prognostic factors in the decision-making of pre-hepatectomy chemotherapy for colorectal cancer hepatic metastasis: The priority of hepatectomy. *World J. Surg. Oncol.* **2018**, *16*, 24. [CrossRef]
3. Vatandoust, S.; Price, T.J.; Karapetis, C.S. Colorectal cancer: Metastases to a single organ. *World J. Gastroenterol.* **2015**, *21*, 11767–11776. [CrossRef] [PubMed]
4. Van Cutsem, E.; Nordlinger, B.; Adam, R.; Köhne, C.; Pozzo, C.; Poston, G.; Ychou, M.; Rougier, P.; European Colorectal Metastases Treatment Group. Towards a pan-European consensus on the treatment of patients with colorectal liver metastases. *Eur. J. Cancer* **2006**, *42*, 2212–2221. [CrossRef] [PubMed]
5. Loree, J.M.; Kopetz, S. Recent developments in the treatment of metastatic colorectal cancer. *Ther. Adv. Med. Oncol.* **2017**, *9*, 551–564. [CrossRef] [PubMed]
6. Saltz, L.B.; Cox, J.V.; Blanke, C.; Rosen, L.S.; Fehrenbacher, L.; Moore, M.J.; Maroun, J.A.; Ackland, S.P.; Locker, P.K.; Pirotta, N.; et al. Irinotecan plus fluorouracil and leucovorin formetastatic colorectal cancer. *Irinotecan Study Group. N. Engl. J. Med.* **2000**, *343*, 905–914. [CrossRef]
7. Venook, A.P.; Niedzwiecki, D.; Lenz, H.J.; Innocenti, F.; Fruth, B.; Meyerhardt, J.A.; Schrag, D.; Greene, C.; O'Neil, B.H.; Atkins, J.N.; et al. Effect of first-line chemotherapy combined with cetuximab or bevacizumab on overall survival in patients with KRAS wild-type advanced or metastatic colorectal cancer: A randomized clinical trial. *JAMA* **2017**, *317*, 2392–2401. [CrossRef]
8. Jin, Z.; Hubbard, J.M. Optimizing biologic sequencing in metastatic colorectal cancer: First line and beyond. *Curr. Oncol.* **2019**, *26*, S33–S42. [CrossRef]
9. Dekker, E.; Tanis, P.J.; Vleugels, J.L.A.; Kasi, P.M.; Wallace, M.B. Colorectal cancer. *Lancet* **2019**, *394*, 1467–1480. [CrossRef]
10. Rawla, P.; Sunkara, T.; Barsouk, A. Epidemiology of colorectal cancer: Incidence, mortality, survival, and risk factors. *Prz. Gastroenterol.* **2019**, *14*, 89–103. [CrossRef] [PubMed]
11. Molinari, C.; Marisi, G.; Passardi, A.; Matteucci, L.; Maio, G.; Ulivi, P. Heterogeneity in colorectal cancer: A challenge for personalized medicine? *Int. J. Mol. Sci.* **2018**, *19*, 3733. [CrossRef]
12. Raimondi, C.; Nicolazzo, C.; Gradilone, A.; Gianni, G.; Falco, E.; Chimenti, I.; Varriali, E.; Hauch, S.; Plappert, L.; Cortesi, E.; et al. Circulating tumor cells: Exploring intratumor heterogeneity of colorectal cancer. *Cancer Biol. Ther.* **2014**, *15*, 496–503. [CrossRef]
13. Sastre, J.; Maestro, M.L.; Gómez-España, A.; Rivera, F.; Valladares, M.; Massuti, B.; Benavides, M.; Gallén, M.; Marcuello, E.; Abad, A.; et al. Circulating tumor cell count is a prognostic factor in metastatic colorectal cancer patients receiving first-line chemotherapy plus bevacizumab: A Spanish Cooperative Group for the Treatment of Digestive Tumors study. *Oncologist* **2012**, *17*, 947–955. [CrossRef] [PubMed]
14. Nakamura, Y.; Yoshino, T. Clinical utility of analyzing circulating tumor DNA in patients with metastatic colorectal cancer. *Oncologist* **2018**, *23*, 1310–1318. [CrossRef]
15. Punt, C.J.; Koopman, M.; Vermeulen, L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. *Nat. Rev. Clin. Oncol.* **2017**, *14*, 235–246. [CrossRef] [PubMed]
16. Turano, M.; Delrio, P.; Rega, D.; Cammarota, E.; Polverino, A.; Duraturo, E.; Izzo, P.; Rosa, M. Promising colorectal cancer biomarkers for precision prevention and therapy. *Cancers (Basel)* **2019**, *11*, 1932. [CrossRef]
17. Baek, D.H.; Kim, G.H.; Song, G.A.; Han, I.S.; Park, E.Y.; Kim, H.S.; Jo, H.J.; Ko, S.H.; Park, D.Y.; Choo, Y. Clinical potential of circulating tumor cells in colorectal cancer: A prospective study. *Clin. Transl. Gastroenterol.* **2019**, *10*, e00055. [CrossRef]
18. Kolenčík, D.; Shishido, S.N.; Pitule, P.; Mason, J.; Hicks, J.; Kuhn, P. Liquid Biopsy in Colorectal Carcinoma: Clinical Applications and Challenges. *Cancers (Basel)* **2020**, *12*, 1376. [CrossRef]
19. Salvianti, F.; Pazzagli, M.; Pinzani, P. Single circulating tumor cell sequencing as an advanced tool in cancer management. *Expert Rev. Mol. Diagn.* **2016**, *16*, 51–63. [CrossRef]
20. Bidard, F.C.; Mathiot, C.; Delaloge, S.; Brain, E.; Giachetti, S.; Cremoux, P.; Marty, M.; Pierga, J.-Y. Single circulating tumor cell detection and overall survival in nonmetastatic breast cancer. *Ann. Oncol.* **2010**, *219*, 729–733. [CrossRef] [PubMed]
21. Cohen, S.J.; Punt, C.J.; Iannotti, N.; Saidman, B.H.; Sabbath, K.D.; Gabrail, N.Y.; Picus, J.; Morse, M.; Mitchell, E.; Miller, M.C.; et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **2008**, *26*, 3213–3221. [CrossRef]
22. Cristofanilli, M.; Budd, G.T.; Ellis, M.J.; Matera, J.; Miller, M.C.; Reuben, J.M.; Doyle, G.V.; Allard, W.J.; Terstappen, L.W.M.M.; Hayes, D.F.; et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* **2004**, *351*, 781–791. [CrossRef]

23. de Bono, J.S.; Scher, H.I.; Montgomery, R.B.; Parker, C.; Miller, M.C.; Tissing, H.; Doyle, G.V.; Terstappen, L.W.M.M.; Pienta, K.J.; Raghavan, D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin. Cancer Res.* **2008**, *14*, 6302–6309. [CrossRef]
24. ClinicalTrials.gov. Available online: https://clinicaltrials.gov/ (accessed on 18 February 2021).
25. Marcuello, M.; Vymetalkova, V.; Neves, R.P.L.; Duran-Sanchon, S.; Vedeld, H.M.; Tham, E.; Dalum, G.; Flügen, G.; Garcia-Barberan, V.; Fijneman, R.J.; et al. Circulating biomarkers for early detection and clinical management of colorectal cancer. *Mol. Aspects Med.* **2019**, *69*, 107–122. [CrossRef]
26. Souza E Silva, V.; Chinen, L.T.; Abdallah, E.A.; Damascena, A.; Paludo, J.; Chojniak, R.; Dettino, A.L.A.; de Mello, C.A.L.; Alves, V.S.; Fanello, M.F. Early detection of poor outcome in patients with metastatic colorectal cancer: Tumor kinetics evaluated by circulating tumor cells. *Oncol. Targets Ther.* **2016**, *9*, 7503–7513. [CrossRef]
27. Buzz, C.; Pop, V.V.; Buiga, R.; Sur, D.; Samasca, G.; Aldea, C.; Lupan, I. Circulating tumor cells in clinical research and monitoring patients with colorectal cancer. *Oncotarget* **2018**, *9*, 24561–24571. [CrossRef]
28. Eslami-S, Z.; Cortés-Hernández, L.E.; Alix-Panabières, C. Epithelial Cell Adhesion Molecule: An Anchor to Isolate Clinically Relevant Circulating Tumor Cells. *Cells* **2020**, *9*, 1836. [CrossRef]
29. Abdallah, E.A.; Fanelli, M.F.; Souza E Silva, V.; Netto, M.C.M.; Gasparini Junior, J.L.; Araújo, D.V.; Ocea, L.M.M.; Buim, M.E.C.; Tariki, M.S.; Alves, V.S.; et al. MRP1 expression in CTCs confers resistance to irinotecan-based chemotherapy in metastatic colorectal cancer. *Int. J. Cancer* **2016**, *139*, 890–898. [CrossRef]
30. Buim, M.E.; Fanelli, M.F.; Souza, V.S.; Romero, J.; Abdallah, E.A.; Mello, C.A.; Alves, V.; Ocea, L.M.M.; Mingues, N.B.; Barbosa, P.N.; et al. Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer. *Cancer Biol. Ther.* **2015**, *16*, 1289–1295. [CrossRef]
31. Abdallah, E.A.; Fanelli, M.F.; Buim, M.E.; Netto, M.C.M.; Gasparini Junior, J.L.; Souza e Silva, V.; Dettino, A.L.A.; Mingues, N.B.; Romero, J.V.; Ocea, L.M.M.; et al. Thymidylate synthase expression in circulating tumor cells: A new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients. *Int. J. Cancer* **2015**, *137*, 1397–1405. [CrossRef]
32. Eisenhauer, E.A.; Therasse, P.; Bogaerts, J.; Schwartz, L.H.; Sargent, D.; Ford, R.; Dancey, J.; Arbuck, S.; Gwyther, S.; Mooney, M.; et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur. J. Cancer* **2009**, *45*, 228–247. [CrossRef]
33. Krebs, M.G.; Hou, J.; Sloane, R.; Lancashire, L.; Priest, L.; Nonaka, D.; Ward, T.H.; Backen, A.; Clack, G.; Hughes, A.; et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. *J. Thorac. Oncol.* **2012**, *7*, 306–315. [CrossRef]
34. Lausen, B.; Schumacher, M. Maximally selected rank statistics. *Biometrics* **1992**, *48*, 73–85. [CrossRef]
35. Cox, D.R. Regression models and life-tables (with discussion). *J. R. Stat. Soc. B* **1972**, *34*, 187–220.
36. Schoenfeld, D. Partial residuals for the proportional hazards regression model. *Biometrika* **1982**, *69*, 239–241. [CrossRef]
37. Grambsch, P.M.; Therneau, T.M. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika* **1994**, *81*, 515–526. [CrossRef]
38. Nikolouzakis, T.K.; Vassilopoulou, L.; Fragkiadaki, P.; Sapsakos, T.M.; Papadakis, G.Z.; Spandidos, D.A.; Tsatsakis, A.M.; Tsiaoussis, J. Improving diagnosis, prognosis and prediction by using biomarkers in CRC patients. (Review). *Oncol. Repogy* **2018**, *39*, 2455–2472. [CrossRef]
39. Taieb, J.; Jung, A.; Sartore-Bianchi, A.; Peeters, M.; Seligmann, J.; Zaanan, A.; Burdon, P.; Montagut, C.; Laurent-Puig, P. The evolving biomarker landscape for treatment selection in metastatic colorectal cancer. *Drugs* **2019**, *79*, 1375–1394. [CrossRef]
40. Xie, Y.; Chen, Y.; Fang, J. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct. Target. Ther.* **2020**, *5*, 22. [CrossRef]
41. Modest, D.P.; Pant, S.; Sartore-Bianchi, A. Treatment sequencing in metastatic colorectal cancer. *Eur. J. Cancer* **2019**, *109*, 70–83. [CrossRef]
42. Vogel, A.; Hofheinz, R.D.; Kubicka, S.; Arnold, D. Treatment decisions in metastatic colorectal cancer—Beyond first and second line combination therapies. *Cancer Treat. Rev.* **2017**, *59*, 54–60. [CrossRef] [PubMed]
43. Pellino, G.; Gallo, G.; Pallante, P.; Capasso, R.; De Stefano, A.; Maretto, I.; Malapelle, U.; Qtu, S.; Nikolaou, S.; Barina, A.; et al. Noninvasive biomarkers of colorectal cancer: Role in diagnosis and personalised treatment perspectives. *Gastroenterol. Res. Pract.* **2018**, *2018*, 2397863. [CrossRef]
44. Tol, J.; Koopman, M.; Miller, M.C.; Tibbe, A.; Cats, A.; Creemers, G.J.M.; Vos, A.H.; Nagtegaal, I.D.; Terstappen, L.W.M.M.; Punt, C.J.A. Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. *Ann. Oncol.* **2010**, *21*, 1006–1012. [CrossRef] [PubMed]
45. Tan, Y.; Wu, H. The significant prognostic value of circulating tumor cells in colorectal cancer: A systematic review and meta-analysis. *Curr. Probl. Cancer* **2018**, *42*, 95–106. [CrossRef] [PubMed]
46. Yang, J.; Song, P.; Zhou, G. A study on the correlations of MRP-1 expression with the pathogenesis and prognosis of colorectal cancer. *J. BUON* **2019**, *24*, 84–90. [PubMed]

Anexo 6 - Artigo do estudo publicado

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cells

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Article

Molecular and Dynamic Evaluation of Proteins Related to Resistance to Neoadjuvant Treatment with Chemoradiotherapy in Circulating Tumor Cells of Patients with Locally Advanced Rectal Cancer

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Abstract: The heterogeneity of response to neoadjuvant chemoradiotherapy (NCRT) is still a challenge in locally advanced rectal cancer (LARC). The evaluation of thymidylate synthase (TYMS) and RAD23 homolog B (RAD23B) expression in circulating tumor cells (CTCs) provides complementary clinical information. CTCs were prospectively evaluated in 166 blood samples (63 patients) with LARC undergoing NCRT. The primary objective was to verify if the absence of RAD23B/TYMS in CTCs would correlate with pathological complete response (pCR). Secondary objectives were to correlate CTC kinetics before (C1)/after NCRT (C2), in addition to the expression of transforming growth factor-β receptor I (TGF-βRI) with survival rates. CTCs were isolated by ISET and evaluated by immunocytochemistry (protein expression). At C1, RAD23B was detected in 54.1% of patients with no pCR and its absence in 91.7% of patients with pCR ($p = 0.014$); $TYMS^-$ was observed in 90% of patients with pCR and $TYMS^+$ in 51.7% without pCR ($p = 0.057$). Patients with CTC2 > CTC1 had worse disease-free survival (DFS) ($p = 0.00025$) and overall survival (OS) ($p = 0.0036$) compared with those with CTC2 ≤ CTC1. TGF-βRI expression in any time correlated with worse DFS ($p = 0.059$). To conclude, RAD23B/TYMS and CTC kinetics may facilitate the personalized treatment of LARC.

Keywords: locally advanced rectal carcinoma; neoadjuvant chemoradiation; liquid biopsy; circulating tumor cell; RAD23B

1. Introduction

Colorectal carcinoma is the second most common cause of death in the USA and 30% of new cases are rectal carcinoma (RC) [1,2]. At diagnosis, about half of all patients

with RC are diagnosed with locally advanced rectal carcinoma (LARC), which comprises, according to the TNM classification, tumors in clinical stages II and III, defined as cT3 or cT4 and/or presence of regional lymph nodes compromised by the tumor [3,4]. The standard of care for patients with cT3/T4 or N+ is neoadjuvant chemoradiation (NCRT) followed by total mesorectal excision (TME) based on the results of the German Rectal Cancer Study CAO/ARO/AIO-94 [5]. Despite significant decrease in local recurrence rates from 10% to 5%, distant metastasis and treatment morbidity are still a major problem [6,7].

More recently, two phase III trials demonstrated an improvement in disease free survival (DFS) for patients treated with total neoadjuvant therapy (TNT) when compared to conventional NCRT [8,9]. Distal rectal carcinoma is even more challenging since the great majority of patients are eventually submitted to rectal amputation and definitive colostomy, generating a high social and psychological impact. As a result, those patients treated with NCRT that present complete clinical response could be watched and have a sphincter sparing approach [10,11].

The development of accurate tools to better select patients to more aggressive therapy is paramount in rectal carcinoma. Given the need to stratify patients into responders and non-responders to NCRT prior to its onset, improving the selection of those most likely to obtain a pathological complete response (pCR), several studies have evaluated possible biomarkers, which allow the dynamic monitoring of the disease [12,13]. However, the results are still controversial with limitations for use in clinical practice.

Liquid biopsy represents a promising tool, in the prognostic evaluation and therapeutic resistance, as circulating tumor DNA (ctDNA) and/or circulating tumor cells (CTCs) can be used in a punctual and/or dynamic way during treatment, complementing the evaluation of the disease [14–16]. Sun et al. demonstrated in a study with 115 patients with LARC that CTCs detection is a powerful tool to assess and predict response to NCRT [17]. In a preliminary study with 30 patients, we showed that thymidylate synthase (TYMS) and RAD23 homolog B (RAD-23B) expression in CTCs before and after NCRT could predict tumor response in LARC patients [18].

Numerous previous studies, in both experimental and clinical trials, associated the overexpression of TYMS with possible resistance to fluoropyrimidine-based chemotherapy in various types of cancer [19,20]. In rectal cancer, previous studies have demonstrated that the low expression TYMS gene correlates with the pathological response to 5-Fluorouracil (5-FU)-based NCRT [21,22].

The identification of LARC patients with resistance to radiotherapy is still an obstacle. RAD23 homolog B (RAD23B) is a DNA repair protein, which is the reason why some studies correlate its expression with resistance to ionizing radiation [23,24]. RAD23B expression is elevated in tumors specimens [23,25], but in rectal cancer, so far, there are no studies that evaluate this possible marker for prediction of response.

Furthermore, understanding the tumor microenvironment formed by interactions among tumor cells, immune system, endothelial cells and cytokines is essential to determine possible therapeutic strategies and prognostic evaluation [26]. The expression of programmed cell death ligand-1 (PD-L1) in rectal cancer is still poorly studied, but some authors have shown that radiotherapy can stimulate its expression, although its prognostic/predictive impact is still unknown [27–30]. The transforming growth factor-β (TGF-β) is one of the numerous cytokines found in the tumor microenvironment and plays an important role in the tumor progression, increased angiogenesis and suppression of the immune response [31]. Thus, evaluating PD-L1 and TGF-β receptor I (TGF-βRI) expression during treatment may be significant for prognostic/prediction and better therapeutic definition.

Finally, given the first results previously presented (Troncarelli et al., 2019), we followed and expanded the sampling of these patients as an attempt to strengthen these results and structure ideas for future clinical trials that incorporate liquid biopsy in the risk stratification and classification of tumors, customizing the best therapeutic strategy for each patient with LARC.

2. Materials and Methods

2.1. Patient Population and Study Design

This is a single center, prospective, observational study carried out at A.C. Camargo Cancer Center, São Paulo, Brazil, with patients with LARC. Inclusion criteria were rectal adenocarcinoma, located up to 8 cm from dentate line by flexible proctoscopy. LARC was classified according to the TNM classification: stage cT3-T4 and/or N+, as defined by magnetic resonance imaging (MRI). The pre-treatment evaluation included a pelvic MRI and a computed tomography (CT) scan of the chest and abdomen, evaluated by a multidisciplinary team. Those patients considered candidates for NCRT by institutional protocol followed by total mesorectal resection (TME) were included. Patients submitted to different neoadjuvant regimens, as short course radiation therapy or TNT were excluded from this analysis. Other inclusion criteria were normal hematological parameters and normal renal function. Exclusion criteria were use of anticoagulation and presence of distant metastasis.

For the study, three serial blood samples were collected. The first sample was collected at the moment of patient inclusion (baseline) before the start of NCRT (collection 1, C1). The second sample was performed after the end of NCRT and before surgery (collection 2, C2), and the third sample was performed between 2 to 4 weeks after surgery (collection 3, C3). Venous blood was collected from the antecubital vein and these samples were stored in the room temperature for a maximum of 6h before analysis (Figure 1).

The primary outcome of this study was to determine whether the absence of RAD23B and TYMS expression in CTC would correlate with pCR to NCRT and thereby identify possible responders to treatment. The secondary outcomes were to assess the CTC kinetics between baseline (C1) and after NCRT (C2) in addition to the evaluation of the immune response markers expression such as TGF-βRI and PD-L1 with recurrence and survival rates.

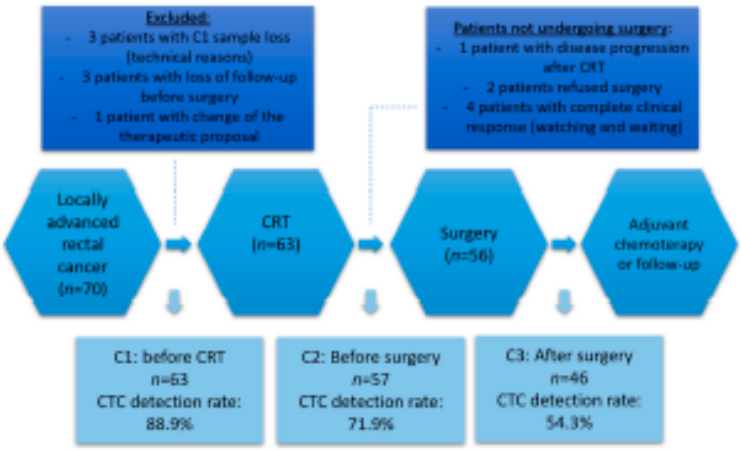

Figure 1. Flowchart showing the timing of each CTC collection (C1, C2 and C3) and its rate of detection during the study and the reasons for excluding patients (at C1) and not undergoing surgical approach (at C2). Abbreviations: CRT, chemoradiotherapy; CTC, circulating tumor cell.

2.2. Treatment

Patients were treated following the therapeutic recommendations of the institutional protocol. All patients received neoadjuvant radiation therapy with a conformed three-dimensional (3D) technique with 45 Gy in 25 fractions to the pelvis. In addition, a boost of 5.4 Gy was administered to the primary tumor and involved lymph nodes in three fractions, for a total of 50.4 Gy applied in 28 fractions. Chemotherapy regimens consisted of

intravenous administration of 5-FU with a dose of 1000 mg/m^2 on days 1 to 5 during weeks 1 and 5 of radiation therapy; or oral capecitabine administered at a dose of 1650 mg/m^2/d during the entire radiation treatment period, depending on the choice of the attending physician. TME surgery were performed about 8–12 weeks after completion NCRT.

For initial assessment of all patients, the clinical examination included digital rectal examination, evaluation of routine laboratory tests such as complete blood count (CBC), liver and kidney function, carcinoembryonic antigen (CEA) level and CA 19.9, colonoscopic examination including biopsy, CT of the chest plus abdomen and pelvic MRI.

2.3. Ethics

This prospective study was approved by the local ethics committee (2141/15C). Written informed consent was obtained from all patients prior to study enrollment. This study was registered at ClinicalTrials.gov (NCT: 02979470).

2.4. Statistical Analysis

Initially, the baseline characteristics were expressed as absolute and relative frequencies for qualitative variables and as the median, minimum and maximum for quantitative variables. In order to evaluate a possible association of qualitative variables in relation to the response (Tumor Regression Grade), the independence test (Fisher's exact test, chi-square test or chi-square test with Yates' continuity correction) was applied to the data.

The univariable and multivariable logistic regression models were fitted to the data in order to identify possible independent factors for the occurrence of the response, in which the measure of association is given by the odds ratio and the respective 95% confidence interval. The independent variables with $p < 0.20$ in the univariable logistic regression model were selected for the multivariable model. The Hosmer–Lemeshow test was applied to evaluate the goodness of fit for logistic regression models. No imputation method was used for missing data.

Regarding the analyzes in which the time until death or recurrence are of interest, the survival analysis approach was used. The survival function was estimated using the Kaplan–Meier (1958) estimator and the estimated survival curves were compared using the log-rank test. In addition, a Cox proportional hazards regression model (Cox, 1972) was fitted to the data to describe the relationship between the independent variables with the time until death or recurrence. For the recurrence outcome, all variables with a $p < 0.20$ in the univariable Cox regression models were considered in the multivariable Cox regression model, while for the outcome of death, due the limitation of the number of events, we selected the variables chosen for the outcome of relapse, also considering their clinical relevance for this outcome. In all fitted models, the assumption of proportionality was assessed using Schoenfeld's residuals (Schoenfeld, 1982; Grambsch and Therneau, 1994) and in all analyses we found evidence that the effect of covariates is constant over time, thus justifying the use of the Cox regression model.

The level of significance adopted was 5% in all analyses. Thus, results whose $p < 0.05$ are considered statistically significant. The software R version 3.6 was used in all analyses.

2.5. Assessment of Preoperative Clinical Response

The evaluation of the clinical response was determined by comparative analysis of the baseline with the physical, endoscopic examination and image at C1 with the preoperative (C2). Clinical response assessment was made within 8–10 weeks after the completion of chemoradiation. In addition, we evaluated the pathological response in comparison to the clinical staging established by baseline images. All parameters for radiological evaluation of the response (patient follow-up images) were obtained at the institution where the study was conducted and reviewed by a radiologist with experience in colorectal tumors.

2.6. Assessment of Pathological Response

The anatomopathological examination of the surgical material of patients submitted to TME was performed to assess the histological type, the tumor differentiation, the pathological stage of the TNM (ypTNM) and the degree of pathological regression of the tumor. The changes observed after NCRT were based on the tumor regression grading (TRG) system, divided into 4 categories according to the guidelines published by American Joint Committee on Cancer (AJCC) and the College of American Pathologists (CAP), with the adaptation of a 3-category classification scheme proposed by Ryan [32,33]. The TRG can be classified into 4 degrees: TRG 0 with no viable neoplastic cells, complete response; TRG 1 represents a moderate response, with rare neoplastic cells isolated or in small groups; TRG 2 is characterized by a minimal response with residual neoplasia with fibrosis; TRG 3 indicates absence of pathological response with extensive residual neoplasia, minimal necrosis. All material was subjected to review by a single pathologist with experience in gastrointestinal pathology, who was blinded to the analysis of CTCs image and analysis.

In addition, we evaluated the pathological response according to the TNM pathological staging (ypTNM) in comparison with the clinical staging established by baseline images to determine the downstaging obtained with the NCRT and, thus, patients were classified into: complete pathological response (ypT0N0), partial pathological (patients with downstaging, but still with residual disease) and without pathological response (without modification of pathological staging when compared to clinical staging).

2.7. Isolation, Quantification and Protein Expression Analysis of CTCs

To isolate and analyze CTCs, we used ISET® (Isolation by SizE of Tumors cells, Rarecells, Paris, France). Briefly, EDTA tubes (8.0 mL BD Vacutainer, Franklin Lakes, NJ, USA) were used to collect peripheral blood samples from patients and were homogenized at room temperature for up to 6 h. Then, they were prepared as described previously (Troncarelli et al., 2019). After filtration, ISET membranes were stored at −20 °C until analysis.

To evaluate protein expression in CTCs, selected spots from the ISET membranes were subjected to a 24-well dual immunocytochemistry (ICC) assay (Polink DS-RR-Hu/Ms A Kit; GBI Labs, Bothell, WA, USA) with the following antibodies: anti-RAD23B (1:100 CSB-PA019260LA01HU; CusaBio, Wuhan, People's Republic of China), anti-TYMS (1:230 WH0007298M1; Sigma-Aldrich, St. Louis, MO, USA), anti-CD45 (1:200 HPA000440; Sigma-Aldrich); anti-PD-L1 (1:300 Ab205921; Abcam, Cambridge, MA, USA), and anti-TGF-βRI (1:100 CSB-PA061850; Cusabio, Wuhan, People's Republic of China. Briefly, the first step was to perform an antigen retrieval using an Antigen Retrieval Solution (Dako, Santa Clara, CA, USA). Following, cells were hydrated with 1 × Tris-buffered saline (TBS), 10 min and permeabilized with Triton X-100 (5 min). Rinsing was done with 1 × TBS and incubation to block endogenous peroxides with 3% hydrogen peroxide in the dark (15 min). Cells were incubated with primary antibodies diluted in 10% fetal calf serum in TBS (overnight). Primary antibody signals were amplified with rabbit horseradish peroxidase (HRP) polymer (GBI Labs) for 30 min and then, for 10 min with 3,3-diaminobenzidine (DAB). After amplification, an incubation with second antibody was made (2 h). Following, we added a rabbit AP polymer (GBI Labs) for 30 min, and GBI-Permanent Red (GBI Labs) for 10 min. Cells were staining with hematoxylin and examined in light microscopy (Research System Microscope BX61; Olympus, Waltham, MA, USA) (Figure 2). CTCs were counted per 1 mL blood, as previously described with statistical methods by Krebs et al. [34] and characterized according to 5 criteria: visible cytoplasm; nucleus size > 18 µm, negativity for CD45 staining; hyperchromatic and irregular nuclei; a nuclear to cytoplasm ratio > 80% [35]. It is important to take in consideration that we used at least 4 ISET spots to make our CTCs counts and that we collected at least 8 mL of blood of each patient. Therefore, the CTC counts are made per mL of blood, but more than 4mL were evaluated for each patient. CTCs were considered positive for the abovementioned antibodies expression if at least one cell was found staining in a spot. We emphasize that when CTC = 0, at any time, it was not possible to evaluate protein expression.

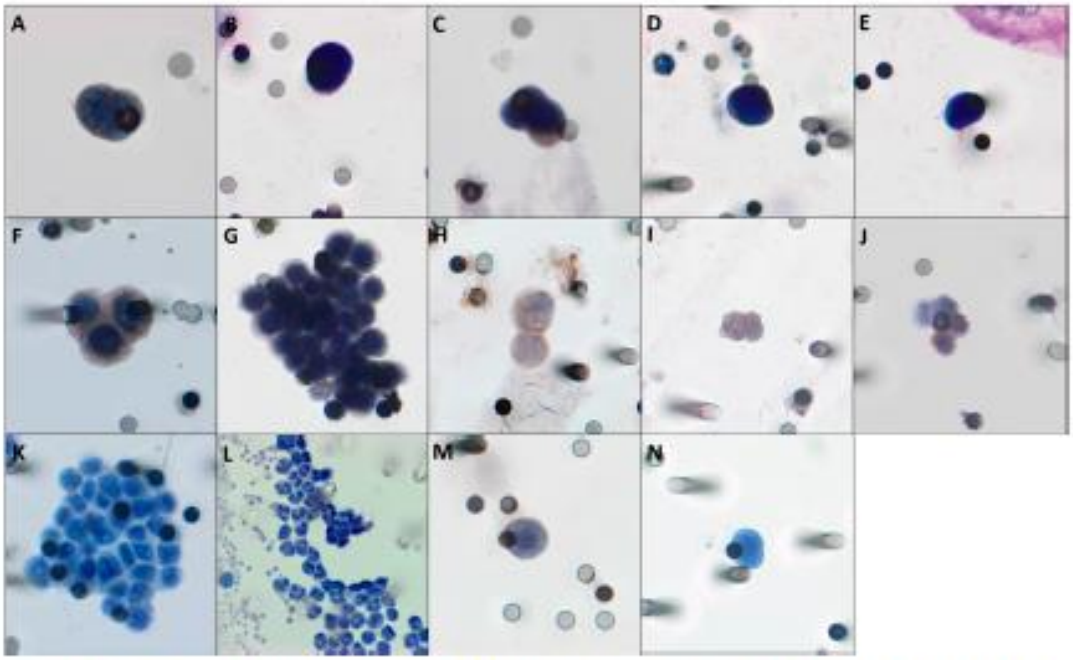

Figure 2. Photomicrographs of CTCs isolated from LARC patients, stained with anti-PD-L1 (**A**), anti-RAD-23B (**B**), anti-TGF-βRI (**C**) and anti-TYMS (**D**). In (**E**), a CTC with hematoxylin, without any antibody staining. Following, in boxes (**F**–**H**), positive controls (cell lineages, that accordingly to Protein Atlas (https://www.proteinatlas.org/ accessed on 13 December 2016) express the studied antibodies, spiked in healthy individual blood). In (**F**), FADU lineage, expressing PD-L1. In box (**G**), HCT-8 cells expressing RAD23B. In (**H**), A549 cells with TGF-βRI staining; in (**I**,**J**), leucocytes staining for TYMS and CD45, respectively. In boxes (**K**,**M**,**N**), negative controls (cell lineages, that accordingly to Protein Atlas (https://www.proteinatlas.org/ accessed on 13 December 2016) do not express or express very low levels of the studied antibodies, spiked in healthy individual blood). In (**K**), PC3 lineage without any expression of PD-L1. In (**M**), MCF7 without TGF-βRI staining and in (**N**), U87MG without TYMS expression. In (**L**), leucocytes from healthy individuals without RAD23B staining. For negative control of CD45, we used A549 lineage.

2.8. Survival Outcomes

In patients undergoing NCRT followed by surgery, the evaluation for adjuvant chemotherapy was performed by the attending physician. The chemotherapy regimen, if indicated, was defined by the team responsible for the patient. For these patients, the beginning of the follow-up was defined at the end of the adjuvant treatment.

In addition, for patients with no indication of adjuvant therapy or for patients who, after the end of NCRT, were not operated on due to evidence of a complete clinical response and, therefore, followed the "Watch and Wait" (WW) strategy, follow-up was initiated at this time.

The follow-up of all patients in the study consisted of clinical, laboratory and imaging assessments every three months.

Patients were followed up from the moment of the first CTC collection (C1) until the moment of death or last follow-up. Loss of follow-up was defined as the patient's absence on two consecutive visits, with no outcome information after this period.

The cCR was defined as clinical disappearance (clinical examination with digital rectal examination), radiological (CT and/or pelvic MRI) and rectoscopy with the appearance of macroscopic disease associated with negative biopsy results in areas suspected or previously affected by the disease.

2.9. Prognosis Evaluation

All enrolled patients were followed until death or until the last follow-up date. Locoregional recurrence was defined as tumor recurrence of lymphatic vessels, anastomosis or adjacent organs. Distant metastasis was defined as the spread of the tumor out of the pelvic cavity. Disease-free survival (DFS) was calculated from the day of surgery (for patients undergoing tumor resection) or the day of evidence of cCR (biopsy and images; for patients who opted for organ preservation) until progression disease (distant metastasis or locoregional recurrence) or death. Overall survival (OS) was calculated from the date of the anatomopathological diagnosis until death or the date of the last follow-up.

3. Results

3.1. Demographic and Clinical Variables

From 2016 to 2020, a total of 166 blood samples were obtained from 63 patients with LARC undergoing treatment. A total of 70 patients were accrued and seven were excluded (three patients with problems in the CTC1 sample, three patients with loss of follow-up before surgery and one patient with modification of the therapeutic proposal). In addition, 7 patients out of the 63 who collected CTC1 (C1) and underwent NCRT did not undergo surgery (one patient with disease progression after NCRT, two patients refused surgery and four patients with a complete clinical response (cCR) being proposed the strategy of WW) (Figure 1).

The clinical features are reported in Table 1. Mean age was 56 years (range 34–92). Thirty-six patients were male (57.1%) and 27 female patients (42.9%). Most patients (80.9%; $n = 51$) had good performance status (ECOG 0) in the inclusion of the study. Moderately differentiated tumors (85.7%; $n = 54$ patients) were found in the diagnosis biopsy.

Table 1. Clinical-pathological aspects, epidemiology, and treatment of 63 patients with locally advanced rectal cancer undergoing neoadjuvant treatment.

Clinical Features	N	%
Total number of patients	63	100
Age (years) at recruitment		
Median	56	
Range (min–max)	34–92	
Gender		
Male	36	57.1
Female	27	42.9
ECOG Performance Status		
0	51	80.9
1	12	19.1
Tumor differentiation		
Well differentiated	7	11.1
Moderately differentiated	54	85.7
Poorly differentiated	2	3.2
Distance of tumor from dentate line (cm)		
<4	34	54
4–8	29	46
Median	4	
Range (min-max)	0.5–8	
Clinical Tumor Stage (cT)		
cT1 or cT2	6	9.5
cT3	51	81
cT4	6	9.5
Clinical Nodal Stage (cN)		
cN0	5	7.9
cN1	38	60.3
cN2	20	31.8

Table 1. *Cont.*

Clinical Features	N	%
CEA in diagnosis (ng/mL; *n* = 61)		
>3	32	52.5
≤3	29	47.5
Ca 19.9 in diagnosis (U/mL; *n* = 55)		
>37	10	18.2
≤37	45	81.8
Neoadjuvant Chemotherapy		
Capecitabine	29	46
5-Fluorouracil (5-FU)	34	54
Pathological Tumor Stage (pT; *n* = 56)		
ypT0-ypT2	34	60.7
ypT3-ypT4	22	39.3
Pathological Nodal Stage (pN; *n* = 56)		
ypN0	45	80.4
ypN1-ypN2	11	19.6
Degree of tumor regression (TRG) (*n* = 56)		
0	13	23.2
1	17	30.4
2	18	32.1
3	7	12.5
Unknown	1	1.8
Pathological Complete Response (*n* = 56)		
Yes	13	23.2
No	43	76.8
MMR (*mismatchrepair*)		
Proficient	38	60.3
Deficient	0	0
Unknown	25	39.7
Adjuvant Chemotherapy		
Yes	40	63.5
No	23	36.5
Locoregional failure and distant metastasis		
Yes	22	34.9
No	41	65.1

Abbreviations: CEA, carcinoembryonic antigen; ECOG: Eastern Cooperative Oncology Group.

At the time of inclusion, 81% (*n* = 51) of the patients had clinical T3 and 60.3% (*n* = 38) clinical N1 according to the eighth edition of AJCC cancer staging manual. Carcinoembryonic antigen (CEA) levels >3.0 ng/mL were found in 52.5% (*n* = 31) and ≤3.0 ng/mL in 47.5% (*n* = 29) patients.

Regarding NCRT, all patients underwent the same radiotherapy protocol. Concerning chemotherapy, 54% (*n* = 34) of patients were submitted to infusional 5-FU and 46% (*n* = 29) to capecitabine.

Out of the 63 patients in the study, 4 had pCR response and of the 56 patients who underwent surgery after NCRT, 23.2% (*n* = 13) had pCR. Of the patients who underwent surgery in relation to the Degree of Tumor Regression (TRG), 23.2% were classified as TRG 0, 30.4 % as TRG 1, 31.1% as TRG 2 and 32.1% as TRG 3.

After surgery, 63.5% (*n* = 40) of the patients underwent adjuvant treatment with chemotherapy. In the follow-up of all patients in the study, 34.9% (*n* = 22) presented locoregional failure and distant metastasis. The main site of metastasis were lung 61.9% (*n* = 13) and liver 42.8% (*n* = 9). Palliative chemotherapy was given to 63.6% (*n* = 14) of patients and 50% (*n* = 11) underwent local treatment with surgery for metastasis resection.

Median follow-up was 32.33 months (95% confidence interval, 28.88–35.78). The mean for DFS was 32.15 months (95% confidence interval, 27.31–36.99) with 22 events and for OS, 46.83 months (95% confidence interval, 44.07–49.59) with 9 events. The median for DFS and OS was not reached.

3.2. CTCs Count

Of the 63 patients in the study, 166 samples were collected for isolation and quantification of CTCs; 63 samples at baseline (C1), 57 samples at first follow-up (C2) and 46 samples at second follow-up (C3) (Figure 1).

The detection rates of CTCs at different time points were: 88.9%, with a median of 2.0 (0–9.0 CTCs/mL) of blood at C1; 71.9% with a median of 0.6 (0–6.3 CTCs/mL) at C2 and at the time C3 was 54.3% with a median of 0.3 (0–4.6 CTCs/mL).

3.3. Protein Expression in CTCs

The expression of RAD23B, TYMS, PD-L1 and TGF-βRI was evaluated in this order of priority according to the primary objective of the study at each time of collection (C1, C2 and C3) and the availability of cells (CTCs count > 0) in the sample and in the spots. The expression of RAD23B was evaluated in 56 samples in the first collection (of the 63 samples collected at C1, 7 samples had CTC = 0), in 41 samples at C2 (out of 57 samples 16 had CTC = 0) and 25 samples at C3 (from 46 collected, 21 had CTC = 0). At C1, 40% of patients (n = 22) had RAD23B positive and 60% (n = 34) negative. For C2 and C3, 39% (n = 16) and 0% (n = 0) of patients showed positive expression for RAD23B, respectively. TYMS expression was evaluated in a similar way to the expression of RAD23B in the same number of samples at each moment and was positive in 35% (n = 20) at C1, 39% (n = 16) at C2 and 0% (n = 0) at C3.

For TGF-βRI expression evaluation, 49 samples were available at C1 (of the 63 samples collected, 7 samples with CTC = 0 and 7 samples with no spot available), 17 samples at C2 (of the 57 samples collected, 16 with CTC = 0 and 24 without available spot) and only two samples at C3 (out of 46 samples, 21 with CTC = 0 and 23 without spot). TGF-βRI positive expression found in 20% (n = 10) of patients at C1, 18% (n = 3) at C2 and 0% (n = 0) at C3.

Finally, for PD-L1 analysis, 41 samples (out of 63 samples, 7 with CTC = 0 and 15 with no available spot) were made at C1, 39 samples at C2 (out of 57 samples, 16 samples with CTC = 0 and 2 samples without spot) and 25 samples at C3 (out of 46 samples, 21 samples with CTC = 0 and 17 samples with no spot for marking). PD-L1 expression was found in 19% (n = 8) of patients at C1, in no patient at C2 and in 16% (n = 4) at C3 (Figure 2).

3.4. Correlation between Clinical Characteristics, CTCs Kinetics and Protein Expression in CTCs with Pathological Response

3.4.1. Clinical Characteristics

For patients with pCR or TRG 0 (23.2%; n = 13/56), 69.2% (n = 9) were female (p = 0.07), moderately differentiated adenocarcinomas (p = 0.09), CEA ≤ 3.0 (p = 0.11) and clinical T3 (p = 0.06); 61.5% (n = 8) of patients with pCR carried medium rectum tumors (p = 0.57) and underwent neoadjuvant treatment with 5-FU (p = 0.99).

In the univariable logistic regression model, pCR was found more in females in relation to males (Odds Ratio (OR) 4.050; 95% confidence interval (CI), 1.064–15.409; p = 0.04), in cT3 in relation to cT2, (OR 0.083; 95% CI, 0.008–0.899; p = 0.04). However, in the multivariable logistic regression model, there was no significant difference for sex and clinical tumor staging (Table 2).

3.4.2. CTCs Kinetics

For patients who had a CTC2 count (C2) higher than a CTC1 count (C1), only one patient (8.3%) had pCR and 6 (15.8%) showed no pCR (p = 0.99). Thus, when comparing patients with unfavorable kinetics (CTC2 > CTC1) with those with favorable kinetics (CTC2 ≤ CTC1), by univariable logistic regression model, there was a tendency of reduction in the chance of complete response (OR, 0.485; 95% CI, 0.052–4.487; p = 0.52), although without statistical significance (Table 2).

Table 2. Univariable and multivariable logistic regression models for complete pathological response.

	Category	Univariable Logistic Regression Model			Multivariable Logistic Regression Model		
		OR	95% CI	*p*	OR	95% CI	*p*
Gender	Male	1.0			1.0		
	Female	4.050	1.064–15.409	0.040	3.180	0.626–16.163	0.163
CEA (ng/mL)	≤3.0	1.0			1.0		
	>3.0	0.329	0.087–1.247	0.102	0.346	0.065–1.858	0.216
Clinical Tumor Stage	cT2	1.0			1.0		
	cT3	0.083	0.008–0.899	0.041	0.568	0.032–10.081	0.700
	cT4	0.067	0.003–1.509	0.089	0.936	0.020–42.968	0.973
Chemotherapy	5-FU	1.0					
	Capecitabine	0.919	0.257–3.292	0.897	—	—	—
RAD23B CTC1	Negative	1.0			1.0		
	Positive	0.077	0.009–0.661	0.019	0.064	0.006–0.751	0.029
TYMS CTC1	Negative	1.0					
	Positive	0.821	0.208–3.239	0.779	—	—	—
CTC2 > CTC1	No	1.0	0.052–4.487	0.524	—	—	—
	Yes	0.485					

Abbreviations: CTC, circulating tumor cells; CI (95%), confidence interval; OR, odds ratio; CEA, carcinoembryonic antigen; cT, clinical tumor stage; 5-FU, 5-fluorouracil.

3.4.3. RAD23B and TYMS Expression

ICC expression of RAD23B at C1 was detected in only one patient with pCR and in 54.1% ($n = 20$) of patients with no pCR. However, in 91.7% of patients with pCR ($p = 0.014$) RAD23B was absent. In the C2 collection, after the end of neoadjuvant with chemotherapy and radiotherapy, the absence of RAD23B expression correlated with the presence of pCR. In the second collection, of the 13 patients with pCR, 10 showed no RAD23B expression in the CTC (100%) and three patients had CTC = 0 (without the possibility to assess the RAD23B expression). On the other hand, for patients who did not obtain pCR with NCRT, 51.7% dissipation the expression of RAD23B in CTC at C2 ($p = 0.06$). Therefore, the absence of RAD23B expression at C1 and at C2 correlated directly with pCR, helping in the management of these patients (Figure 3).

In the univariable (OR 0.077 (95% CI, 0.009–0.661); $p = 0.019$) and multivariable (OR 0.064 (95% CI, 0.006–0.751); $p = 0.029$) logistic regression models for pCR, we observed that RAD23B expression was associated with decreased chance of pCR as compared to no expression (Table 2).

In addition, 4 out of 63 patients had a complete clinical response and underwent an organ preservation strategy and, therefore, did not undergo surgery after the end of the NCRT. In these 4 patients, all did not show expression of RAD23B at C1 ($p \leq 0.001$) and at C2, three patients with the presence of CTC had no expression of RAD23B ($p \leq 0.001$) reinforcing the correlation of the response to NCRT with the absence of RAD23B. One patient at C2 did not have isolated CTC, making it impossible to assess RAD23B expression.

We evaluated the expression of TYMS by ICC in CTCs at C1. We evidenced that among patients with pCR, 33.3% ($n = 4$) had positive expression of TYMS. For patients who did not obtain a pCR, 37.8% ($n = 14$) had positive expression ($p = 0.99$). In the univariable logistic regression model, the presence of TYMS expression showed no correlation (OR 0.821; 95% CI, 0.208–3.239; $p = 0.77$) with the absence of pCR (Table 2). On the other hand, when we evaluated the expression of TYMS after NCRT (C2), 90% of patients with pCR did not have TYMS expression ($p = 0.057$). By univariable logistic regression model, the presence

of TYMS expression in the CTC presented OR of 0.119 (95% CI, 0.013–1.064; $p = 0.057$) for pCR.

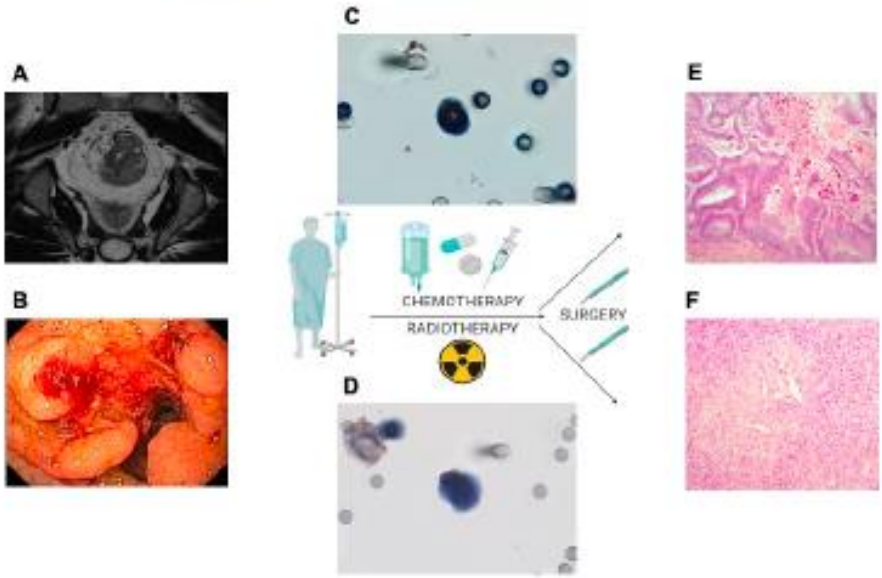

Figure 3. Illustration of a patient with LARC (A,B) evidenced in baseline imaging exams with RAD23B positive in CTC (C) with no response TRG 3 (E) and RAD23B negative on CTC (D) with pCR TRG 0 (F). (A): Rectal lesion on pelvic MRI with extension to perirectal fat with compromised mesorectal lymph nodes; (B): Rectoscopy with evidence of ulcerative injury to the rectum; (C): CTC collected at the time C1 or C2 with positive RAD23B; (D): CTC collected at the time C1 or C2 with negative RAD23B; (E): Anatomopathological examination of the surgical material indicates absence of pathological response with extensive residual neoplasia (TRG 3); (F): Anatomopathological examination of the surgical material with evidence of pCR (TRG 0). Abbreviations: LARC, locally advanced rectal carcinoma; RAD23B, RAD23 homolog B; CTC, circulating tumor cell; TRG, tumor regression grading; MRI, magnetic image resonance; C1, collection 1; C2, collection 2.

In the combined analysis of RAD23B and TYMS expression in CTCs at C1, we showed that among patients with pCR, 66.7% ($n = 8$) had no RAD23B/ TYMS expression; for patients with no pCR, 29.7% ($n = 11$) had RAD23B/ TYMS positive and 24.3 % ($n = 9$) had negative TYMS and positive RAD 23 B ($p = 0.029$). In the second collection (C2), for patients with pCR, 90% ($n = 9$) had no RAD23B/TYMS staining and for patients with no pCR, 41.4% ($n = 12$) had RAD23B/TYMS staining ($p = 0.029$). These data reinforce the correlation between the absence of RAD23B/TYMS expression in CTCs and the response before and after NCRT.

3.5. *Correlation between CTCs Kinetics and Protein Expression in CTCs with DFS and OS*

Patients who presented unfavorable CTC kinetics during neoadjuvant treatment (CTC2 > CTC1) had worse DFS (median 9.11 months; 95% CI 6.21–12.00) compared with those with favorable kinetics (CTC2 ≤ CTC1) (median 33.85 months; 95% CI 28.63–30.07) ($p = 0.00025$). The unfavorable kinetics also correlated with poor OS. For patients with CTC2 > CTC1, the median OS was 35.57 months (95% CI 26.10–45.05) and for patients with favorable kinetics the mean OS was 47.74 months (95% CI 44.93–50.56) ($p = 0.0036$) (Figure 4).

In the univariable analysis for patients with CTC2 > CTC1, we demonstrated worse DFS and OS, respectively (hazard ratio (HR) 7.042; 95% CI, 2.111–23.491; $p = 0.001$) and (HR 4.954; 95% CI, 1.141–21.504; $p = 0.033$). Cox's multivariable regression model suggests

that CTC2 > CTC1 remained an independent predictor of DFS and OS, respectively (HR 12.463; 95% CI, 2.998–51.811; $p = 0.001$) and (HR 5.503; 95% CI, 1.255–24.122; $p = 0.024$) (Tables 3 and 4).

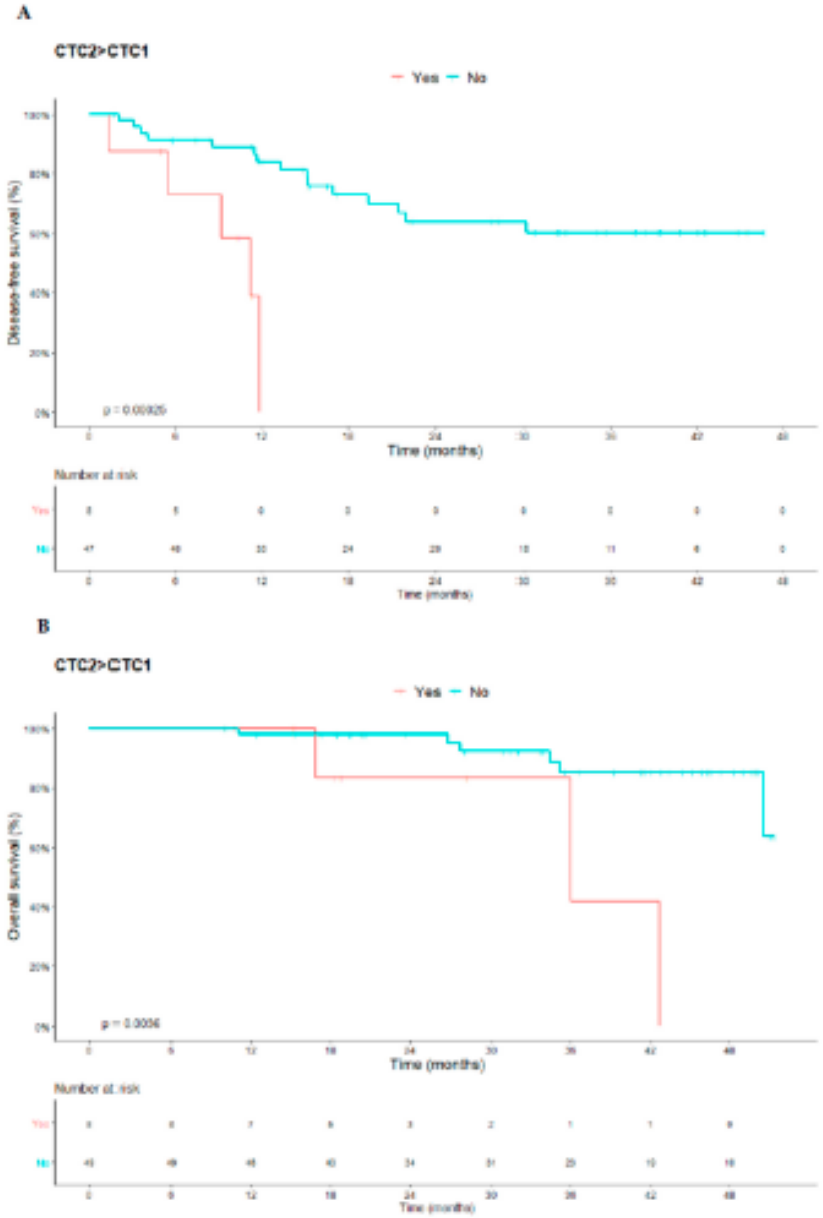

Figure 4. Analysis of DFS and OS of patients with LARC in relation to the kinetics of CTCs (CTC2 > CTC1). (**A**): DFS median of 9.11 months of patients with unfavorable kinetics (CTC2 > CTC1) versus favorable kinetics (CTC2 < CTC1) with a median of 33.85 months; (**B**): OS of patients with unfavorable kinetics (CTC2 > CTC1) with a median of 35.57 months versus favorable kinetics (CTC2 ≤ CTC1) with a median of 47.74 months. Abbreviations: CTCs, circulating tumor cells; DFS, disease-free survival; OS, overall survival; LARC, locally advanced rectal carcinoma.

Table 3. Estimate of the parameters of the univariable and multivariable Cox regression models for disease-free survival of patients with LARC undergoing NCRT.

	Category	Univariable Cox Regression Model			Multivariable Cox Regression Model		
		HR	95% CI	*p*	HR	95% CI	*p*
pCR	No	1.0			1.0		
	Yes	0.138	0.018–1.029	0.053	0.388	0.146–1.032	0.058
CEA (ng/mL)	≤3.0	1.0			—		
	>3.0	2.527	0.969–6.590	0.058	—	—	—
CTC2 > CTC1	No	1.0			1.0		
	Yes	7.042	2.111–23.491	0.001	12.463	2.998–51.811	0.001
RAD23B CTC1	Negative	1.0			—		
	Positive	1.655	0.651–4.206	0.290	—	—	—
TGF-βRI combined CTC	Negative	1.0			1.0		
	Positive	2.314	0.944–5.676	0.067	2.235	0.830–6.018	0.111
Adjuvant Chemotherapy	No	1.0			—	—	—
	Yes	0.537	0.217–1.348	0.185	—	—	—

Abbreviations: CI (95%), confidence interval; HR, hazard ratio; CTC, circulating tumor cells; CEA, carcinoembryonic antigen; pCR, complete pathological response; TGF-βRI combined CTC, Expression of TGF-βRI in CTC1 and/or CTC2 and/or CTC3.

Table 4. Estimate of the parameters of the univariable and multivariable Cox regression models for overall survival of patients with LARC undergoing NCRT.

	Category	Univariable Cox Regression Model			Multivariable Cox Regression Model		
		HR	95% CI	*p*	HR	95% CI	*p*
pCR	No	1.0			1.0		
	Yes	0.440	0.054–3.579	0.443	0.378	0.081–1.771	0.217
CEA (ng/mL)	≤3.0	1.0			—		
	>3.0	2.576	0.498–13.333	0.259	—	—	—
CTC2 > CTC1	No	1.0			1.0		
	Yes	6.502	1.531–27.607	0.011	5.503	1.255–24.122	0.024
RAD CTC1	Negative	1.0			—		
	Positive	1.602	0.375–6.847	0.525	—	—	—
Adjuvant Chemotherapy	No	1.0			—		
	Yes	0.379	0.097–1.476	0.162	—	—	—
TGF-βRI combined CTC	Negative	1.0			1.0		
	Positive	2.117	0.567–7.908	0.265	1.849	0.397–8.611	0.433

Abbreviations: CI (95%), confidence interval; HR, hazard ratio; CTC, circulating tumor cell; CEA, carcinoembryonic antigen; pCR, complete pathological response; TGF-βRI combined CTC, Expression of TGFβ-RI in CTC1 and/or CTC2 and/or CTC3.

3.6. *Protein Expression in CTCs x DFS and OS*

We evaluated the expression of RAD23B, TYMS, PD-L1 and TGF-βRI at each time of collection according to the availability of cells and/or spots of ISET membranes for the immunocytochemistry.

The positive expression of RAD23B at C1 and C2 collection, although shown to be correlated with the absence of a pCR, had no significant impact on the prognosis. At C1, patients with positive expression for RAD23B had a mean DFS of 30.20 months versus 34.87 months for patients without expression of RAD23B ($p = 0.28$) and at time C2 it

was 25.83 months for patients with positive expression versus 32.64 months for negative ($p = 018$). For OS at C1, patients with positive RAD23B showed 46.79 months versus 47.98 months with negative expression ($p = 0.52$) and at C2 it was 43.65 months for patients with positive expression versus 47.49 for patients with negative RAD23B ($p = 0.23$). For univariable analysis of the expression of RAD23B in the first collection, DFS (HR 1.655; 95% CI, 0.651–4.206; $p = 0.29$) and OS (HR 1.602; 95% CI, 0.375–6.847; $p = 0.52$) showed a tendency towards a worse prognosis. The expression of TYMS showed no difference in DFS and OS.

A combined analysis of the three collections (C1, C2 and C3) was made for the expression of PD-L1 and TGF-βRI, that is, we analyzed the positivity for these proteins at any time. From the total of 166 samples from the three moments of the study, we were able to evaluate the combined expression for PD-L1 and TGF-βRI, respectively, in 63% ($n = 105$) and 41% ($n = 68$) samples.

The combined expression of PD-L1 in CTCs showed a trend to better DFS of 32.91 months for patients with positive expression for PD-L1 and 30.77 months for patients with no expression ($p = 0.32$). For OS, patients with negative expression for PD-L1 had OS of 46.40 months versus 45.07 months for those with positive expression ($p = 0.64$).

When we evaluated the combined expression of TGF-βRI, we noticed a tendency that its expression in the CTC at any time of the treatment (C1 or C2 or C3) could be correlated with a worse DFS, that is, patients who presented positivity to the expression of TGF-βRI had an average DFS of 22.36 months versus 34.47 months for patients with negative expression ($p = 0.059$) (Figure 5). For OS, patients with positive expression had an average of 44.34 months versus 47.27 months for patients without expression of TGF-βRI on CTC ($p = 0.25$).

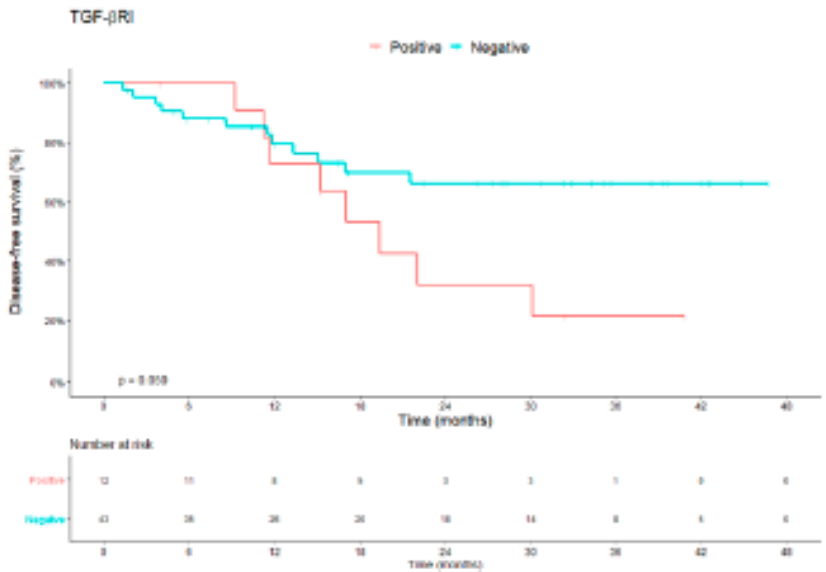

Figure 5. DFS analysis of patients with LARC for combined TGF-βRI expression in CTC during treatment (C1, C2 or C3). Median DFS of 22.36 months for patients with positive expression for TGF-B and 34.47 months for patients with negative expression ($p = 0.059$). Abbreviations: CTCs, circulating tumor cells; DFS, disease-free survival; LARC, locally advanced rectal carcinoma.

4. Discussion

In the present study, we reinforce the previously published data, which favors the applicability of CTC in the management of the LARC patients. Unfortunately, the response to NCRT is heterogeneous, with only about 20% of patients with LARC undergoing NCRT

presenting a pCR and 40% of patients with no response or minimal response [36–39]. In our study, we found this heterogeneity, with 13 patients (23.2%) with pCR and 44.6% ($n = 25$) with poor response (TRG 2 and 3) to NCRT.

Important studies have demonstrated the relevance of pCR, as an outcome that must be achieved in NCRT, as it is associated with good DFS and 5-year OS [11,40]. For patients with pCR, especially for tumors of the distal rectum, the WW strategy is quite attractive and safe [10,41]. However, the clinical response presents poor correlation with the pathological response [42–44]. Therefore, in addition to endoscopy and imaging, new tools are need to more accurately predict response. In view of the preservation strategy, there is a rationale for treatment intensification with the aim of increasing the response to treatment. The OPRA study [45], whose preliminary results were presented at ASCO 2020, demonstrated that TNT is important for WW strategy.

Ionizing Radiation (IR) is an important pillar of NCRT for patients with LARC, with the aim of providing cell damage and death [23,46]. However, not all tumor cells have radiosensitivity, since there are mechanisms capable of effectively repairing their damaged DNA [47,48]. Therefore, understanding these mechanisms is fundamental to search for possible biomarkers of resistance to this therapeutic strategy. Thus, as we have already described, the expression of RAD23B could be studied as a possible biomarker for resistance to IR [18]. Here, we demonstrated RAD23B expression was associated with a lower chance of pCR compared to no expression in the univariable analysis (OR 0.077; 95% CI; 0.009–0.661; $p = 0.019$) and multivariable logistic regression (OR 0.064 (95% CI), 0.006–0.751; $p = 0.029$). Despite the wide confidence interval, mainly justified by the number of patients involved in the regression model ($n = 47$), this data demonstrates that the presence of RAD23B in CTC1 implies a 93.6% chance of not presenting pCR to NCRT. In other words, in our cohort, the absence of RAD23B expression at the baseline and/or after NCRT reinforces the correlation with the pCR. In the 13 patients with pCR and in the four patients with cCR, the absence of RAD23B expression correlated with response to treatment. As far as we know, our results are the first to show the possible role of this molecule in rectum cancer and its analysis in CTCs can be an additional tool to complement the response assessment exams already adopted in medical practice, reinforcing the evidence of cCR for WW strategy.

On the other hand, for non-responders, the standard NCRT must be reevaluated in order to avoid unnecessary toxicities or, eventually, establish new treatment strategies capable of optimizing the response of these patients, such as the adoption of more intense treatments according to the reasoning proposed by the TNT's strategy [7,49]. In view of this, numerous efforts with studies evaluating the intensification of NCRT are underway and the recently published RAPIDO study and the PRODIGE 23 phase III study reinforced the importance of the TNT strategy with the addition of chemotherapy (FOLFOX/CAPOX or FOLFIRINOX) in increasing the rate of pCR and reducing about 7% of distant metastases [8,9]. However, we reinforce with our results that the risk stratification must be improved and the incorporation of the liquid biopsy in these studies must be considered, in order to avoid overtreatment for some patients. In addition, the identification of markers such as RAD23B and TYMS in CTCs could identify patients who are radioresistant to standard NCRT treatment. With this information, we could, in future clinical trials, assess whether in these patients we could omit radiotherapy. This could minimize possible adverse effects provided by NCRT and thereby surrogate for more intense chemotherapy regimens for patients whose CTCs express RAD23B.

Another relevant point of our study was that we demonstrated the importance of analyzing the kinetics of CTCs during NCRT. Previous studies of our group had already demonstrated this relevance for patients with metastatic colorectal cancer [50,51], that is, the quantification of CTC at different times helps us to stratify patients with unfavorable kinetics (CTC2 > CTC1) versus favorable kinetics (CTC2 ≤ CTC1). However, for localized disease, we still have few studies. Despite failing to demonstrate a difference with the pCR, patients with favorable kinetics tend to evolve with a greater chance of pCR compared to those with unfavorable one. Therefore, CTC kinetics can be applied in larger studies to

corroborate the prediction of response. Considering DFS and OS, patients who presented unfavorable kinetics (CTC2 > CTC1), that is, there was an increase in the quantification of CTC during treatment with NCRT, presented inferior DFS and OS in relation to those with favorable kinetics (CTC2 ≤ CTC1). Therefore, in view of these results, we could discuss to intensify treatment with chemotherapy for these patients. For the analysis of TGF-βRI and PD-L1 we had difficulties due to the limited number of available spots from ISET membranes to evaluate these markers in patients CTCs. Even with these limitations, we were able to demonstrate that the expression of TGF-βRI during treatment may, despite the lack of statistical significance, be related to worse prognosis. This result corroborates studies that describe that higher levels of TGF-βRI may be indicative of distant metastases with worse outcomes. Maybe our work may pave the way for future trials to better evaluate TGF-βRI in rectal cancer. Concerning PD-L1, the clinical benefit of the blocking this molecule in some tumors, such as rectal cancer, still remains uncertain. Some previous studies demonstrate that the expression of PD-L1 in the tumor during neoadjuvant treatment can be modified and have a prognostic impact [27–29]. Here, unfortunately due, probably, the limitation of the samples, we could not demonstrate any prognostic difference. However, we observed that the positive expression of PD-L1 in CTCs occurs dynamically with treatment, as of 105 samples evaluated, 8, 0 and 4 were positive for PD-L1 at C1, C2 and C3, respectively. Therefore, by demonstrating in our study the possibility of dynamically analysis of this expression in CTCs, we could evaluate new therapeutic perspectives for these patients with the use of immunotherapy guided by the expression of PD-L1 in in these cells, suggesting a NCRT strategy combined with blockade of the PD-1/PD-L1 pathway. At the American Society of Clinical Oncology (ASCO) congress, that happened in June 2021, Salvatori et al. reported the addition of Avelumab (anti-PD-L1) in patients with LARC undergoing NCRT. Although they did not search for PDL-1 expression in tumor sample, the authors demonstrated that the combination of NCRT with Avelumab showed promising activity and a viable safety profile that should be explored in future clinical studies [52].

We are aware of the limitations of our study, mostly, the small sample size, but we believe that despite of this, our results must be explored in future clinical trials, as we showed that the evaluation of the expression of RAD23B and TYMS associated with the evaluation of CTC kinetics may facilitate the personalized treatment of rectal cancer. In Table 5 we describe the main points that strengthen our results and our limitations. Despite the important impact on local control provided by NCRT, remote recurrence is still a problem and around 20–30% of patients have distant metastases after curative treatment [6,53], so, novel tools like liquid biopsy are very promising.

Table 5. Description of the main points that strengthen the study and its limitations.

Strengths of the Future Perspectives	Limitations
Molecular and dynamic evaluation of proteins in circulating tumor cells allow complementary predictive and prognostic evaluation;	The proteins TGF-βRI and PD-L1 were not evaluated in all CTC samples;
CTC kinetics (favorable kinetics versus unfavorable kinetics) enable prognostic assessment during treatment and thus evaluate more intensive treatment regimens;	Probably because of the limitation of the number of samples, we could not demonstrate any prognostic difference in relation to PD-L1 expression.
The absence of RAD23B expression in CTCs may identify radiosensitive patients and improve selection for sphincter preservation strategy;	

Table 5. *Cont.*

Strengths of the Future Perspectives	Limitations
The expression of TGF-βRI in CTCs may assist the prognostic evaluation of locally advanced rectal cancer (LARC) patients and be used as target molecule for future clinical trials;	Sample size, volume of blood collected and availability of ISET spots should be explored in future clinical trials for the incorporation of liquid biopsy in the therapeutic management of patients with rectal cancer undergoing neoadjuvant treatment.
Serial blood collection during treatment makes the dynamic analysis of the expression of PD-L1 in CTCs feasible and, therefore, permit to evaluate the possible incorporation of immunotherapy in the neoadjuvant treatment of LARC.	

Author Contributions: V.S.e.S.: conception/project, provision of study material or patients, data collection and/or assembly, data analysis, interpretation writing of the manuscript; E.A.A.: conception/project, writing of the manuscript; B.d.C.T.F.: conception/project, provision of study material or patients; A.C.B.: data analysis and interpretation; V.A.G.: provision of study material or patients; D.d.J.F.C.: data analysis and interpretation; A.P.C.R.: data analysis and conception of figures; M.L.G.S.: provision of study material or patients; G.G.M.: data analysis and image evaluation; L.C.L.C.: data analysis and anatomopathological evaluation; V.F.C.: data analysis and interpretation; S.A.J.: conception/design, analysis and interpretation of data; C.A.L.d.M.: conception/design, analysis and interpretation of data; L.T.D.C.: conception/design, analysis and interpretation of data, writing of the manuscript, final approval of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Ethics Committee of ACCamargo Cancer Center (protocol code 2141/15D approved on 26 April 2016).

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

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Conflicts of Interest: The authors declare no potential conflicts of interest.

References

1. Siegel, R.L.; Miller Mph, K.D.; Goding Sauer, A.; Fedewa, S.A.; Butterly, L.F.; Anderson, J.C.; Cercek, A.; Smith, R.A.; Jemal, A. Colorectal cancer statistics, 2020. *CA Cancer J. Clin.* **2020**, *70*, 145–164. [CrossRef]
2. Feeney, G.; Sehgal, R.; Sheehan, M.; Hogan, A.; Regan, M.; Joyce, M.; Kerin, M. Neoadjuvant radiotherapy for rectal cancer management. *World J. Gastrointest.* **2019**, *25*, 4850–4869. [CrossRef] [PubMed]
3. James, D.B.; Mary, K.; Gospodarowicz, C.W. *TNM Classification of Malignant Tumours*, 8th ed.; Wiley-Blackwell: Hoboken, NJ, USA, 2016.
4. Glynne-Jones, R.; Wyrwicz, L.; Tiret, E.; Brown, G.; Rödel, C.; Cervantes, A.; Arnold, D. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **2017**, *28*, iv22–iv40. [CrossRef]
5. Sauer, R.; Becker, H.; Hohenberger, W.; Rodel, C.; Wittekind, C.; Fietkau, R.; Martus, P.; Tschmelitsch, J.; Hager, E.; Hess, C.E.; et al. Preoperative versus Postoperative Chemoradiotherapy for Rectal Cancer. *N. Engl. J. Med.* **2004**, *351*, 1731–1740. [CrossRef] [PubMed]
6. Sakland, A.; Pandey, D.; Patel, S. RAPIDO protocol: A promising approach for high-risk locally advanced rectal cancers. *Lancet Oncol.* **2021**, *22*, 2–3. [CrossRef]
7. Franke, A.J.; Parekh, H.; Starr, J.S.; Tan, S.A.; Iqbal, A.; George, T.J. Total Neoadjuvant Therapy: A Shifting Paradigm in Locally Advanced Rectal Cancer Management. *Clin. Color. Cancer* **2018**, *17*, 1–12. [CrossRef] [PubMed]

8. Bahadoer, R.R.; Dijkstra, E.A.; van Etten, B.; Marijnen, C.A.M.; Putter, H.; Kranenbarg, E.M.-K.; Roodvoets, A.G.H.; Nagtegaal, I.D.; Beets-Tan, R.G.H.; Blomqvist, L.K.; et al. Short-course radiotherapy followed by chemotherapy before total mesorectal excision (TME) versus preoperative chemoradiotherapy, TME, and optional adjuvant chemotherapy in locally advanced rectal cancer (RAPIDO): A randomised, open-label, phase 3 trial. *Lancet Oncol.* **2021**, *22*, 29–42. [CrossRef]
9. Conroy, T.; Bosset, J.-F.; Etienne, P.-L.; Rio, E.; François, É.; Mesgouez-Nebout, N.; Vendrely, V.; Artignan, X.; Bouché, O.; Gargot, D.; et al. Neoadjuvant chemotherapy with FOLFIRINOX and preoperative chemoradiotherapy for patients with locally advanced rectal cancer (UNICANCER-PRODIGE 23): A multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol.* **2021**, *22*, 702–715. [CrossRef]
10. Habr-Gama, A.; Julião, G.P.S.; Perez, R.O. Nonoperative Management of Rectal Cancer. *Hematol. Clin. N. Am.* **2015**, *29*, 135–151. [CrossRef]
11. Sun, W.; Al-Rajabi, R.; Perez, R.O.; Abbasi, S.; Ash, R.; Habr-Gama, A. Controversies in Rectal Cancer Treatment and Management. *Am. Soc. Clin. Oncol. Educ. Book* **2020**, *40*, 136–146. [CrossRef]
12. Ryan, É.J.; Creavin, B.; Sheahan, K. Delivery of Personalized Care for Locally Advanced Rectal Cancer: Incorporating Pathological, Molecular Genetic, and Immunological Biomarkers Into the Multimodal Paradigm. *Front. Oncol.* **2020**, *10*, 1369. [CrossRef]
13. Molinari, C.; Matteucci, F.; Caroli, P.; Passardi, A. Biomarkers and Molecular Imaging as Predictors of Response to Neoadjuvant Chemoradiotherapy in Patients With Locally Advanced Rectal Cancer. *Clin. Color. Cancer* **2015**, *14*, 227–238. [CrossRef] [PubMed]
14. Alix-Panabières, C.; Pantel, K. Liquid Biopsy: From Discovery to Clinical Application. *Cancer Discov.* **2021**, *11*, 858–873. [CrossRef] [PubMed]
15. Heidrich, I.; Ačkar, L.; Mohammadi, P.M.; Pantel, K. Liquid biopsies: Potential and challenges. *Int. J. Cancer* **2021**, *148*, 528–545. [CrossRef]
16. Massihnia, D.; Pizzutilo, E.G.; Amatu, A.; Tosi, F.M.; Ghezzi, S.; Bencardino, K.; Di Masi, P.; Righetti, E.; Patelli, G.; Scaglione, F.; et al. Liquid biopsy for rectal cancer: A systematic review. *Cancer Treat. Rev.* **2019**, *79*, 101893. [CrossRef] [PubMed]
17. Sun, W.; Li, G.; Wan, J.; Zhu, J.; Shen, W.; Zhang, Z. Circulating tumor cells: A promising marker of predicting tumor response in rectal cancer patients receiving neoadjuvant chemo-radiation therapy. *Oncotarget* **2016**, *7*, 69507–69517. [CrossRef]
18. Flores, B.T.; E Silva, V.S.; Abdallah, E.A.; Mello, C.A.; Silva, M.L.G.; Mendes, G.G.; Braun, A.C.; Júnior, S.A.; Chinen, L.T.D. Molecular and Kinetic Analyses of Circulating Tumor Cells as Predictive Markers of Treatment Response in Locally Advanced Rectal Cancer Patients. *Cells* **2019**, *8*, 641. [CrossRef]
19. Aguiar, S.; Lopes, A.; Soares, F.A.; Rossi, M.; Ferreira, F.; Nakagawa, W.; Carvalho, A.; Filho, W. Prognostic and predictive value of the thymidylate synthase expression in patients with non-metastatic colorectal cancer. *Eur. J. Surg. Oncol.* **2006**, *31*, 863–868. [CrossRef]
20. Formentini, A.; Henne-Bruns, D.; Kornmann, M. Thymidylate synthase expression and prognosis of patients with gastrointestinal cancers receiving adjuvant chemotherapy: A review. *Langenbeck's Arch. Surg.* **2004**, *389*, 405–413. [CrossRef]
21. Bottarelli, L.; De' Angelis, G.L.; Azzoni, C.; Di Mario, F.; De' Angelis, N.; Leandro, G.; Fornaroli, F.; Galani, E.; Negri, F. Potential Predictive Biomarkers in Locally Advanced Rectal Cancer Treated with Preoperative Chemo-Radiotherapy. *Acta Biomed.* **2018**, *89*, 102–106. [CrossRef] [PubMed]
22. Kuremsky, J.G.; Tepper, J.E.; McLeod, H.L. Biomarkers for Response to Neoadjuvant Chemoradiation for Rectal Cancer. *Int. J. Radiat. Oncol.* **2009**, *74*, 673–688. [CrossRef]
23. Shen, Y.N.; Bae, I.-S.; Park, G.H.; Choi, H.S.; Lee, K.-H.; Kim, S.H. MicroRNA-196b enhances the radiosensitivity of SNU-638 gastric cancer cells by targeting RAD23B. *Biomed. Pharmacother.* **2018**, *105*, 362–369. [CrossRef]
24. Dantuma, N.P.; Heinen, C.; Hoogstraten, D. The ubiquitin receptor Rad23: At the crossroads of nucleotide excision repair and proteasomal degradation. *DNA Repair* **2009**, *8*, 449–460. [CrossRef]
25. Abbasi, R.; Ramroth, H.; Becher, H.; Dietz, A.; Schmezer, P.; Popanda, O. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in*ERCC5*,*ERCC6*and*RAD23B*but not by polymorphisms in five other nucleotide excision repair genes. *Int. J. Cancer* **2009**, *125*, 1431–1439. [CrossRef]
26. Corrò, C.; Dutoit, V.; Koessler, T. Emerging Trends for Radio-Immunotherapy in Rectal Cancer. *Cancers* **2021**, *13*, 1374. [CrossRef]
27. Boustani, J.; Derangère, V.; Bertaut, A.; Adotevi, O.; Morgand, V.; Charon-Barra, C.; Ghiringhelli, F.; Mirjolet, C. Radiotherapy Scheme Effect on PD-L1 Expression for Locally Advanced Rectal Cancer. *Cells* **2020**, *9*, 2071. [CrossRef]
28. Richter, L.; Jirasek, T.; Dvorak, J.; Cermakova, E.; Rehakova, P.; Bartos, J. The prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression in patients with locally advanced rectal adenocarcinoma. *J. BUON* **2017**, *22*, 875–881.
29. Hecht, M.; Büttner-Herold, M.; Erlenbach-Wünsch, K.; Haderlein, M.; Croner, R.; Grützmann, R.; Hartmann, A.; Fietkau, R.; Distel, L.V. PD-L1 is upregulated by radiochemotherapy in rectal adenocarcinoma patients and associated with a favourable prognosis. *Eur. J. Cancer* **2016**, *65*, 52–60. [CrossRef]
30. Huemer, F.; Kieser, E.; Neureiter, D.; Schlintl, V.; Rinnerthaler, G.; Pagès, F.; Kirilovsky, A.; El Sissy, C.; Igbeder, W.; Singhartinger, F.; et al. Impact of PD-L1 Scores and Changes on Clinical Outcome in Rectal Cancer Patients Undergoing Neoadjuvant Chemoradiotherapy. *J. Clin. Med.* **2020**, *9*, 2775. [CrossRef]
31. Angenete, E.; Langenskiöld, M.; Palmgren, I.; Falk, P.; Öresland, T.; Ivarsson, M.-L. Transforming growth factor beta-1 in rectal tumour, mucosa and plasma in relation to radiotherapy and clinical outcome in rectal cancer patients. *Int. J. Color. Dis.* **2007**, *22*, 1331–1338. [CrossRef]

8. Bahadoer, R.R.; Dijkstra, E.A.; van Etten, B.; Marijnen, C.A.M.; Putter, H.; Kranenbarg, E.M.-K.; Roodvoets, A.G.H.; Nagtegaal, I.D.; Beets-Tan, R.G.H.; Blomqvist, L.K.; et al. Short-course radiotherapy followed by chemotherapy before total mesorectal excision (TME) versus preoperative chemoradiotherapy, TME, and optional adjuvant chemotherapy in locally advanced rectal cancer (RAPIDO): A randomised, open-label, phase 3 trial. *Lancet Oncol.* **2021**, *22*, 29–42. [CrossRef]
9. Conroy, T.; Bosset, J.-F.; Etienne, P.-L.; Rio, E.; François, É.; Mesgouez-Nebout, N.; Vendrely, V.; Artignan, X.; Bouché, O.; Gargot, D.; et al. Neoadjuvant chemotherapy with FOLFIRINOX and preoperative chemoradiotherapy for patients with locally advanced rectal cancer (UNICANCER-PRODIGE 23): A multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol.* **2021**, *22*, 702–715. [CrossRef]
10. Habr-Gama, A.; Julião, G.P.S.; Perez, R.O. Nonoperative Management of Rectal Cancer. *Hematol. Clin. N. Am.* **2015**, *29*, 135–151. [CrossRef]
11. Sun, W.; Al-Rajabi, R.; Perez, R.O.; Abbasi, S.; Ash, R.; Habr-Gama, A. Controversies in Rectal Cancer Treatment and Management. *Am. Soc. Clin. Oncol. Educ. Book* **2020**, *40*, 136–146. [CrossRef]
12. Ryan, É.J.; Creavin, B.; Sheahan, K. Delivery of Personalized Care for Locally Advanced Rectal Cancer: Incorporating Pathological, Molecular Genetic, and Immunological Biomarkers Into the Multimodal Paradigm. *Front. Oncol.* **2020**, *10*, 1369. [CrossRef]
13. Molinari, C.; Matteucci, F.; Caroli, P.; Passardi, A. Biomarkers and Molecular Imaging as Predictors of Response to Neoadjuvant Chemoradiotherapy in Patients With Locally Advanced Rectal Cancer. *Clin. Color. Cancer* **2015**, *14*, 227–238. [CrossRef] [PubMed]
14. Alix-Panabières, C.; Pantel, K. Liquid Biopsy: From Discovery to Clinical Application. *Cancer Discov.* **2021**, *11*, 858–873. [CrossRef] [PubMed]
15. Heidrich, I.; Ačkar, L.; Mohammadi, P.M.; Pantel, K. Liquid biopsies: Potential and challenges. *Int. J. Cancer* **2021**, *148*, 528–545. [CrossRef]
16. Massihnia, D.; Pizzutilo, E.G.; Amatu, A.; Tosi, F.M.; Ghezzi, S.; Bencardino, K.; Di Masi, P.; Righetti, E.; Patelli, G.; Scaglione, F.; et al. Liquid biopsy for rectal cancer: A systematic review. *Cancer Treat. Rev.* **2019**, *79*, 101893. [CrossRef] [PubMed]
17. Sun, W.; Li, G.; Wan, J.; Zhu, J.; Shen, W.; Zhang, Z. Circulating tumor cells: A promising marker of predicting tumor response in rectal cancer patients receiving neoadjuvant chemo-radiation therapy. *Oncotarget* **2016**, *7*, 69507–69517. [CrossRef]
18. Flores, B.T.; E Silva, V.S.; Abdallah, E.A.; Mello, C.A.; Silva, M.L.G.; Mendes, G.G.; Braun, A.C.; Júnior, S.A.; Chinen, L.T.D. Molecular and Kinetic Analyses of Circulating Tumor Cells as Predictive Markers of Treatment Response in Locally Advanced Rectal Cancer Patients. *Cells* **2019**, *8*, 641. [CrossRef]
19. Aguiar, S.; Lopes, A.; Soares, F.A.; Rossi, M.; Ferreira, F.; Nakagawa, W.; Carvalho, A.; Filho, W. Prognostic and predictive value of the thymidylate synthase expression in patients with non-metastatic colorectal cancer. *Eur. J. Surg. Oncol.* **2006**, *31*, 863–868. [CrossRef]
20. Formentini, A.; Henne-Bruns, D.; Kornmann, M. Thymidylate synthase expression and prognosis of patients with gastrointestinal cancers receiving adjuvant chemotherapy: A review. *Langenbeck's Arch. Surg.* **2004**, *389*, 405–413. [CrossRef]
21. Bottarelli, L.; De' Angelis, G.L.; Azzoni, C.; Di Mario, F.; De' Angelis, N.; Leandro, G.; Fornaroli, F.; Galani, E.; Negri, F. Potential Predictive Biomarkers in Locally Advanced Rectal Cancer Treated with Preoperative Chemo-Radiotherapy. *Acta Biomed.* **2018**, *89*, 102–106. [CrossRef] [PubMed]
22. Kuremsky, J.G.; Tepper, J.E.; McLeod, H.L. Biomarkers for Response to Neoadjuvant Chemoradiation for Rectal Cancer. *Int. J. Radiat. Oncol.* **2009**, *74*, 673–688. [CrossRef]
23. Shen, Y.N.; Bae, I.-S.; Park, G.H.; Choi, H.S.; Lee, K.-H.; Kim, S.H. MicroRNA-196b enhances the radiosensitivity of SNU-638 gastric cancer cells by targeting RAD23B. *Biomed. Pharmacother.* **2018**, *105*, 362–369. [CrossRef]
24. Dantuma, N.P.; Heinen, C.; Hoogstraten, D. The ubiquitin receptor Rad23: At the crossroads of nucleotide excision repair and proteasomal degradation. *DNA Repair* **2009**, *8*, 449–460. [CrossRef]
25. Abbasi, R.; Ramroth, H.; Becher, H.; Dietz, A.; Schmezer, P.; Popanda, O. Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in*ERCC5*,*ERCC6*and*RAD23B*but not by polymorphisms in five other nucleotide excision repair genes. *Int. J. Cancer* **2009**, *125*, 1431–1439. [CrossRef]
26. Corrò, C.; Dutoit, V.; Koessler, T. Emerging Trends for Radio-Immunotherapy in Rectal Cancer. *Cancers* **2021**, *13*, 1374. [CrossRef]
27. Boustani, J.; Derangère, V.; Bertaut, A.; Adotevi, O.; Morgand, V.; Charon-Barra, C.; Ghiringhelli, F.; Mirjolet, C. Radiotherapy Scheme Effect on PD-L1 Expression for Locally Advanced Rectal Cancer. *Cells* **2020**, *9*, 2071. [CrossRef]
28. Richter, L.; Jirasek, T.; Dvorak, J.; Cermakova, E.; Rehakova, P.; Bartos, J. The prognostic effect of neoadjuvant chemoradiotherapy on the change of PD-L1 expression in patients with locally advanced rectal adenocarcinoma. *J. BUON* **2017**, *22*, 875–881.
29. Hecht, M.; Büttner-Herold, M.; Erlenbach-Wünsch, K.; Haderlein, M.; Croner, R.; Grützmann, R.; Hartmann, A.; Fietkau, R.; Distel, L.V. PD-L1 is upregulated by radiochemotherapy in rectal adenocarcinoma patients and associated with a favourable prognosis. *Eur. J. Cancer* **2016**, *65*, 52–60. [CrossRef]
30. Huemer, F.; Kieser, E.; Neureiter, D.; Schlintl, V.; Rinnerthaler, G.; Pagès, F.; Kirilovsky, A.; El Sissy, C.; Igbeder, W.; Singhartinger, F.; et al. Impact of PD-L1 Scores and Changes on Clinical Outcome in Rectal Cancer Patients Undergoing Neoadjuvant Chemoradiotherapy. *J. Clin. Med.* **2020**, *9*, 2775. [CrossRef]
31. Angenete, E.; Langenskiöld, M.; Palmgren, I.; Falk, P.; Öresland, T.; Ivarsson, M.-L. Transforming growth factor beta-1 in rectal tumour, mucosa and plasma in relation to radiotherapy and clinical outcome in rectal cancer patients. *Int. J. Color. Dis.* **2007**, *22*, 1331–1338. [CrossRef]

32. Ryan, R.; Gibbons, D.; Hyland, J.M.P.; Treanor, D.; White, A.; Mulcahy, H.E.; O'Donoghue, D.P.; Moriarty, M.; Fennelly, D.; Sheahan, K. Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *Histopathology* **2005**, *47*, 141–146. [CrossRef]
33. Mace, A.G.; Pai, R.K.; Stocchi, L.; Kalady, M.F. American Joint Committee on Cancer and College of American Pathologists Regression Grade. *Dis. Colon Rectum* **2015**, *58*, 32–44. [CrossRef] [PubMed]
34. Krebs, M.; Hou, J.-M.; Sloane, R.S.; Lancashire, L.; Priest, L.; Nonaka, D.; Ward, T.H.; Backen, A.; Clack, G.; Hughes, A.; et al. Analysis of Circulating Tumor Cells in Patients with Non-small Cell Lung Cancer Using Epithelial Marker-Dependent and -Independent Approaches. *J. Thorac. Oncol.* **2012**, *7*, 306–315. [CrossRef]
35. Khoja, L.; Backen, A.; Sloane, R.S.; Menasce, L.; Ryder, D.; Krebs, M.; Board, R.; Clack, G.; Hughes, A.; Blackhall, F.; et al. A pilot study to explore circulating tumour cells in pancreatic cancer as a novel biomarker. *Br. J. Cancer* **2011**, *106*, 508–516. [CrossRef] [PubMed]
36. Martin, S.T.; Heneghan, H.; Winter, D.C. Systematic review and meta-analysis of outcomes following pathological complete response to neoadjuvant chemoradiotherapy for rectal cancer. *Br. J. Surg.* **2012**, *99*, 918–928. [CrossRef] [PubMed]
37. Ryan, J.E.; Warrier, S.K.; Lynch, A.C.; Heriot, A.G. Assessing pathological complete response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer: A systematic review. *Color. Dis.* **2015**, *17*, 849–861. [CrossRef] [PubMed]
38. Maas, M.; Nelemans, P.J.; Valentini, V.; Das, P.; Rödel, C.; Kuo, L.-J.; Calvo, F.A.; García-Aguilar, J.; Glynne-Jones, R.; Haustermans, K.; et al. Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: A pooled analysis of individual patient data. *Lancet Oncol.* **2010**, *11*, 835–844. [CrossRef]
39. Muñoz, R.C.; Cuadros, M.; Zambudio, N.; Segura-Jiménez, L.; Cano, C.; Palma, P. Predictive Biomarkers to Chemoradiation in Locally Advanced Rectal Cancer. *BioMed Res. Int.* **2015**, *2015*, 921436. [CrossRef]
40. Chen, H.; Feng, L.; Li, M.; Ju, H.; Ding, Y.; Lan, M.; Song, S.; Han, W.; Yu, L.; Wei, M.; et al. College of American Pathologists Tumor Regression Grading System for Long-Term Outcome in Patients with Locally Advanced Rectal Cancer. *Oncologist* **2021**, *26*, e780–e793. [CrossRef]
41. Van der Valk, M.J.M.; Hilling, D.; Bastiaannet, E.; Kranenbarg, E.M.-K.; Beets, G.L.; Figueiredo, N.L.; Habr-Gama, A.; Perez, R.O.; Renehan, A.G.; van de Velde, C.J.H.; et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): An international multicentre registry study. *Lancet* **2018**, *391*, 2537–2545. [CrossRef]
42. López-Campos, F.; Martín-Martín, M.; Fornell-Pérez, R.; García-Pérez, J.C.; Dio-Trill, J.; Fuentes-Mateos, R.; López-Durán, S.; Domínguez-Rullán, J.; Ferreiro, R.; Riquelme-Oliveira, A.; et al. Watch and wait approach in rectal cancer: Current controversies and future directions. *World J. Gastroenterol.* **2020**, *26*, 4218–4239. [CrossRef] [PubMed]
43. Caturegli, I.; Molin, M.D.; Laird, C.; Molitoris, J.K.; Bafford, A.C. Limited Role for Routine Restaging After Neoadjuvant Therapy in Locally Advanced Rectal Cancer. *J. Surg. Res.* **2020**, *256*, 317–327. [CrossRef]
44. Seo, N.; Kim, H.; Cho, M.S.; Lim, J.S. Response Assessment with MRI after Chemoradiotherapy in Rectal Cancer: Current Evidences. *Korean J. Radiol.* **2019**, *20*, 1003–1018. [CrossRef]
45. García-Aguilar, J.; Patil, S.; Kim, J.K.; Yuval, J.B.; Thompson, H.; Verheij, F.; Lee, M.; Saltz, L.B.; on behalf of the OPRA Consortium Preliminary results of the organ preservation of rectal adenocarcinoma (OPRA) trial. *J. Clin. Oncol.* **2020**, *38*, 4008. [CrossRef]
46. Hubenak, J.R.; Zhang, Q.; Branch, C.D.; Kronowitz, S.J. Mechanisms of Injury to Normal Tissue after Radiotherapy. *Plast. Reconstr. Surg.* **2014**, *133*, 49e–56e. [CrossRef]
47. Huerta, S.; Gao, X.; Saha, D. Mechanisms of resistance to ionizing radiation in rectal cancer. *Expert Rev. Mol. Diagn.* **2009**, *9*, 469–480. [CrossRef]
48. Allal, A.S.; Kähne, T.; Reverdin, A.K.; Lippert, H.; Schlegel, W.; Reymond, M.-A. Radioresistance-related proteins in rectal cancer. *Proteomics* **2004**, *4*, 2261–2269. [CrossRef]
49. Kast, A.; Abbasi, S.; Handa, S.; Al-Rajabi, R.; Saeed, A.; Baranda, J.; Sun, W. Total Neoadjuvant Therapy vs Standard Therapy in Locally Advanced Rectal Cancer. *JAMA Netw. Open* **2020**, *3*, e2030097. [CrossRef]
50. E Silva, V.S.; Chinen, L.; Abdallah, E.A.; Damascena, A.; Paludo, J.; Chojniak, R.; Dettino, A.; Mello, C.A.L.; Alves, V.S.; Fanelli, M.F. Early detection of poor outcome in patients with metastatic colorectal cancer: Tumor kinetics evaluated by circulating tumor cells. *OncoTargets Ther.* **2016**, *9*, 7503–7513. [CrossRef]
51. Silva, V.; Abdallah, E.; Brito, A.; Braun, A.; Tariki, M.; de Mello, C.; Calsavara, V.; Riechelmann, R.; Chinen, L. Baseline and Kinetic Circulating Tumor Cell Counts Are Prognostic Factors in a Prospective Study of Metastatic Colorectal Cancer. *Diagnostics* **2021**, *11*, 502. [CrossRef] [PubMed]
52. Salvatore, L.; Benst, M.; Corallo, S.; Bergamo, F.; Pellegrini, I.; Rasola, C.; Borelli, B.; Tamburini, E.; Randon, G.; Galuppo, S.; et al. Phase II study of preoperative (PREOP) chemoradiotherapy (CTRT) plus avelumab (AVE) in patients (PTS) with locally advanced rectal cancer (LARC): The AVANA study. *J. Clin. Oncol.* **2021**, *39*, 3511. [CrossRef]
53. Zaborowski, A.; Stakelum, A.; Winter, D.C. Systematic review of outcomes after total neoadjuvant therapy for locally advanced rectal cancer. *Br. J. Surg.* **2019**, *106*, 979–987. [CrossRef] [PubMed]

Apêndice 1 - Termo de Consentimento Livre e Esclarecido (TCLE).

A.C.Camargo Cancer Center

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

NOME DO ESTUDO: IDENTIFICAÇÃO DE PROTEÍNAS E GENES DE FATORES DE TRANSCRIÇÃO RELACIONADOS À RESISTÊNCIA A QUIMIOTERAPIA E RADIOTERAPIA EM CÉLULAS TUMORAIS CIRCULANTES DE PACIENTES COM CÂNCER DE CÓLON E RETO ESTÁDIOS II E III

Investigador Responsável: Dra. Ludmilla T. Domingos Chinen
Nome do (a) Voluntário (a) : _____
RGH:_____

Você está sendo convidado (a) a participar de uma pesquisa.

Por favor, leia cuidadosamente este termo, pois ele informa o que você necessita saber sobre os objetivos deste estudo. Se concordar em tomar parte neste estudo, deverá assinar e datar este termo. A sua assinatura significa que recebeu as informações necessárias e que deseja participar deste estudo.

OBJETIVOS DO ESTUDO

Usar um equipamento laboratorial novo no mercado para isolar células tumorais circulantes (são células que se desprendem do tumor) no sangue periférico de pacientes com câncer de cólon/reto localmente avançados e correlacionar seus níveis com:

- recorrência tumoral local/à distância;

- resposta ao tratamento (verificaremos se há diminuição dos níveis dessas células durante e após o seu tratamento);

- expressão de genes e proteínas de resistência a drogas (ERCC1, resistência a derivados de platina e TYMS, resistência a 5-fluorouracil, tratamentos comumente utilizados), genes de invasão tumoral (TGFβ, MMP2, MMP9), proliferação (Ki-67) e dormência celular (β-gal), que poderiam auxiliar no acompanhamento do câncer;

- sobrevida livre de progressão;

- tempo de vida após o tratamento.

PROCEDIMENTOS DO ESTUDO

Se concordar em participar deste estudo, você será submetido a três coletas de sangue (aproximadamente 10 mL), antes do início do tratamento (cirurgia ou quimioterapia/radioterapia), após este tratamento e aproximadamente a cada 4 meses, até a mudança do protocolo do tratamento ou, se a doença progredir, até a progressão.

Caso haja necessidade, dependendo do tratamento e de seu consentimento, mais coletas de sangue serão realizadas.

Você não precisará vir ao hospital apenas para a coleta desta pesquisa. Contataremos você nas datas que você tiver exames e na maioria das vezes, aproveitaremos as punções venosas (coleta de sangue) dos exames no laboratório de rotina. Caso não tenha exames a ser realizados, contataremos você nos dias que tiver consulta ou outros procedimentos no hospital.

Todo o material coletado (sangue) será esgotado em um processo de filtração. Armazenaremos em freezer apenas o material contido no filtro (células, DNA, RNA, plaquetas e plasma) e analisaremos na pesquisa.

Toda identificação do seu material será feita por códigos para preservar sua confidencialidade.

RISCOS

O seu tratamento será exatamente o mesmo, caso você participe ou não deste estudo. Não haverá danos imediatos ou tardios, que comprometam a sua saúde, salvos possíveis desconfortos e riscos decorrentes da punção venosa, procedimento comum para exames hematológicos.

Os riscos a que você estará sujeito são os riscos inerentes a qualquer punção venosa como: dor local no ato da punção, sangramento no local, hematoma e raramente flebite (infecção na veia puncionada), mas isto será evitado pela limpeza adequada do local de punção e realização do procedimento por profissional capacitado. Apesar de raro, pode ser que ocorra equimose (quando o sangue sai para a pele, resultando em uma mancha azul ou púrpura, redonda não elevada ou irregular) após a coleta de sangue. Caso isso aconteça com você, não há nada a ser feito, a não ser esperar que desapareça (desaparece em até 7 dias).

Os exames realizados em seu sangue não implicarão em nenhuma mudança em seu tratamento ou qualquer conduta médica que esteja sendo realizada ou venha a ser realizada no seu seguimento, uma vez que a pesquisa de CTCs no Brasil ainda é experimental, não havendo nenhum prejuízo para o sucesso das condutas médicas outras aos quais você esteja se submetendo.

Garantimos a assistência integral e gratuita pelo tempo que for necessário em caso de danos decorrentes da pesquisa.

Embora garantindo que seus dados serão identificados por meio de códigos e siglas, existe sempre o risco da perda de confidencialidade entre os investigadores.

BENEFÍCIOS

Não haverá benefícios de qualquer espécie para você, apenas a importância de contribuir para uma pesquisa científica cujos dados obtidos poderão trazer benefícios para pessoas com câncer. A recusa em participar, não acarretará prejuízo na qualidade do seu tratamento. O pesquisador responsável se compromete a suspender a pesquisa imediatamente ao perceber algum risco ou dano à sua saúde, que não previsto neste termo de consentimento.

MÉTODOS ALTERNATIVOS EXISTENTES

Apesar de métodos alternativos serem aprovados e usados na prática clínica em outros países, no Brasil, esses métodos ainda não estão disponíveis.

ACOMPANHAMENTO, ASSISTÊNCIA E RESPONSÁVEIS

Os pesquisadores também se comprometem a dar informação atualizada ao longo do estudo, caso este seja o seu desejo, porém, por se tratar de nova tecnologia, os resultados não poderão indicar cura ou piora do se quadro, pois ainda não temos valores de referência para estabelecer uma comparação. Contato da pesquisadora: Dra. Ludmilla T. Domingos Chinen (2189-5000 ramal 2776).

Caso os achados da pesquisa, fatos ou informações encontradas pelo pesquisador no decorrer da pesquisa sejam considerados de relevância para você ou para comunidades participantes, os mesmos serão divulgados de forma a contribuir com o acompanhamento do câncer, juntamente com os exames de rotina. Garantimos, ainda que os resultados dos exames genéticos não sejam fornecidos a terceiros (como, por exemplo: seguradoras, empregadores, supervisores hierárquicos, entre outros).

CUSTOS

Não haverá qualquer custo ou forma de pagamento para você por sua participação neste estudo.

CARÁTER CONFIDENCIAL DOS REGISTROS

Seus registros médicos poderão ser consultados pela equipe de pesquisadores envolvidos neste estudo. Os dados obtidos pela análise do seu sangue são confidenciais, não sendo divulgada a identificação de nenhum participante ainda que informações do registro médico sejam utilizadas para publicação.

BASES DA PARTICIPAÇÃO

É importante que você saiba que a sua participação neste estudo é completamente voluntária e que você pode recusar-se a participar ou interromper sua participação a qualquer momento, sem penalidades ou perda de benefícios aos quais tem direito.

SALVAGUARDA DE CONFIDENCIALIDADE, SIGILO E PRIVACIDADE

A eventual inclusão dos resultados em publicação científica será feita de modo a manter seu anonimato. Você terá acesso aos seus dados de exames, atendimentos médicos e administração de terapia, quando solicitados.

ESCLARECIMENTOS SOBRE COMPENSAÇÕES OU DANOS RELACIONADOS À PESQUISA

Você não terá nenhum tipo de remuneração ao aceitar participar deste estudo. A pesquisa não envolve nenhuma forma de compensação financeira aos participantes.

ESCLARECIMENTOS SOBRE OUTROS DIREITOS DO PACIENTE SUJEITO À PESQUISA

A sua participação no estudo é voluntária. Você tem o direito de sair do estudo a qualquer momento e por qualquer motivo. Caso venha a abandonar o estudo ou decidir não participar do mesmo, o seu tratamento não será prejudicado. No entanto, se você decidir sair da pesquisa, deverá informar ao seu médico.

INFORMAÇÕES SOBRE NOMES, TELEFONES E ENDEREÇOS PARA CONTATOS

Esclarecimentos para questões sobre os direitos dos participantes na pesquisa e/ou danos relacionados à pesquisa, contatar a pesquisadora Dra. Ludmilla T. Domingos Chinen (2189 5000 ramal 2776), endereço: Rua Professor Antônio Prudente, 211- Liberdade- São Paulo, SP. Se o pesquisador principal não fornecer as informações/esclarecimentos suficientes, por favor, entre em contato com o coordenador do Coordenador do Comitê de Ética em Seres Humanos da Fundação Antônio Prudente-Hospital do Câncer – A.C. Camargo/ SP, cujo horário de funcionamento é de segunda a quinta-feira, das 07h00 às 18h00 e de sexta-feira das 07h00 às 16h00 (Telefone 2189-5000 ramal 5020; endereço: Rua Professor Antônio Prudente, 211- Liberdade- São Paulo, SP).

O Comitê de Ética em Pesquisa é uma comissão composta por médicos, enfermeiras, farmacêuticos, biólogos, psicólogos, fisioterapeutas, pesquisadores e membros da comunidade. Utiliza mecanismos, ferramentas e instrumentos próprios de inter-relação, num trabalho cooperativo que visa, especialmente, à proteção dos participantes de pesquisa do Brasil, de forma coordenada e descentralizada por meio de um processo de acreditação.

O termo é elaborado em duas vias e rubricado em todas as páginas, sendo uma via retida com o pesquisador responsável e outra com a instituição.

Você receberá uma via deste documento. Somente assine este documento se consentir integralmente com seus termos.

GARANTIA DE ESCLARECIMENTOS

Nós lhe estimulamos a fazer perguntas a qualquer momento do estudo. Se tiver perguntas relacionadas aos seus direitos como participante do estudo clínico, também pode contar com uma terceira pessoa imparcial, o Coordenador do Comitê de Ética do Hospital A. C. Camargo.

DECLARAÇÃO DE CONSENTIMENTO E ASSINATURA

Li as informações acima e entendi o propósito deste estudo assim como os benefícios e riscos potenciais da participação no mesmo. Tive a oportunidade de fazer perguntas e todas foram respondidas. Eu, por intermédio deste, dou livremente meu consentimento para participar neste estudo. Entendo que não serei submetido a nenhum exame adicional e não receberei compensação monetária por minha participação neste estudo.

Eu recebi uma via assinada deste formulário de consentimento.

_____ ____/____/_____
Assinatura do (a) voluntário (a) dia mês ano

_____
Nome do (a) voluntário (a) - letra de forma

_____ ____/____/_____
(Assinatura de Testemunha, se necessário) dia mês ano

Eu, abaixo assinado, expliquei completamente os detalhes relevantes deste estudo ao paciente indicado acima e/ou pessoa autorizada para consentir pelo paciente.

_____ ____/____/____
(Assinatura da pessoa que obteve o consentimento) dia mês ano