BIOLOGICAL AND SURGICAL DETERMINANTS IN THE TREATMENT OF RECTAL CANCER

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A thesis submitted in fulfilment of the requirements for the Doctoral Degree in Medicine, in the Specialty of Clinical Research

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LIST OF SCIENTIFIC PUBLICATIONS

The following publications are included in this Thesis:

1. Cravo M, Rodrigues T, Ourô S, Ferreira A, Féria L, Maio R *Management of rectal cancer: Times are changing*. GE Port J Gastroenterol. 2014;21(5):192-200
Original Paper 1, Chapter 2

2. Ourô S, Mourato C, Ferreira MP, Albergaria D, Cardador A, Castro RE, Maio R, Rodrigues CMP *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma*. Front Oncol (2020) Oct 27; 10:577653. DOI: 10.3389/fonc.2020.577653
Original Paper 2, Chapter 3

3. Ourô S, Mourato C, Ferreira MP, Albergaria D, Cardador A, Castro RE, Maio R, Rodrigues CMP *Evaluation of Tissue and Circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer.* (2020) Pharmaceuticals, Sep 14;13(9):246. DOI: 10.3390/ph13090246
Original Paper 3, Chapter 3

4. Ourô S, Albergaria D, Ferreira MP, Costeira B, Roquete D, Ferreira D, Maio R *Transanal total mesorectal excision: 3-year oncological outcomes.* Coloproctol (2020) Oct 28. DOI: 10.1007/s10151-020-02362-y
Original Paper 4, Chapter 4

5. Ourô S**,** Ferreira MP, Roquete P, Maio R, *Transanal versus Laparoscopic Total Mesorectal Excision. Comparative study of long-tem oncological outcomes*. Submitted.
Original Paper 5, Chapter 4

6. Shaikh I, Askari A, Ourô S**,** Warusavitarne J, Athanasiou T, Faiz O *Oncological outcomes of local excision compared with radical surgery after neoadjuvant chemoradiotherapy for rectal cancer: a systematic review and meta-analysis.* Int J Colorectal Dis (2015) 30:19–29. DOI: 10.1007/s00384-014-2045-1.
Original Paper 6, Chapter 4

7. Ourô S, Ferreira MP, Albergaria D, Maio R *Loop ileostomy in rectal cancer surgery: factors predicting reversal and stoma related morbidity.* Langenbecks Arch Surg 2021 May; 406(3): 843-853. DOI: 10.1007/s00423-021-02169-x
Original Paper 7, Chapter 4

CONTRIBUTION OF THE PhD CANDIDATE IN NON-FIRST AUTHOR PUBLICATIONS

In **Original Paper 1, Chapter 2** (Cravo M, Rodrigues T, Ourô S, Ferreira A, Féria L, Maio R. *Management of rectal cancer: Times are changing*. GE Port J Gastroenterol. 2014;21(5):192-200), being the third author of this review on rectal cancer, the PhD candidate performed the literature research related to the surgical approach in rectal cancer, wrote the chapters related to this issue, namely "Surgery", "Local excision methods", "Radical resections", and "Intersphincteric resection". The PhD Candidate also contributed to the writing of all other chapters having reviewed the manuscript entirely.

In **Original Paper 6, Chapter 4** (Shaikh I, Askari A, Ourô S**,** Warusavitarne J, Athanasiou T, Faiz O *Oncological outcomes of local excision compared with radical surgery after neoadjuvant chemoradiotherapy for rectal cancer: a systematic review and meta-analysis* Int J Colorectal Dis (2015) 30:19–29), being the third author of this review and metanalysis, the PhD Candidate performed the literature search using MEDLINE/ PubMed/ Ovid databases and Google Scholar, the data collection, the final definition of the studies to include and partial writing of the manuscript. The document was also entirely review by the PhD Candidate.

PUBLICATIONS IN COLLABORATION WITH TATME REGISTRY

These papers were written in the context of collaborative work on one of the subjects presented in this Thesis (transanal total mesorectal excision) and occurred during the time of the research presented here, not having been included in it.

Incidence and Risk Factors for Anastomotic Failure in 1594 Patients Treated by Transanal Total Mesorectal Excision: Results From the International TaTME Registry. Marta Penna, Roel Hompes, Steve Arnold *et al*, International TaTME Registry Collaborativative Ann Surg 2019 Apr; 269 (4): 700-711; doi: 10.1097 /SLA.0000000000002653 - **Chapter 4**

Predictive Factors and Risk Model for Positive Circumferential Resection Margin Rate After Transanal Total Mesorectal Excision in 2653 Patients With Rectal Cancer. Sapho Roodbeen, FB de Lacy, Susana van Deren *et al*. International TaTME Registry Collaborative Ann Surg 2019 Nov; 270 (5): 884-891. doi: 10.1097/SLA.0000000000003516 - **Chapter 4**

Carbon Dioxide Embolism Associated With Transanal Total Mesorectal Excision Surgery: A Report From the International Registries. Dickson EA, Penna M, *et al*. International TaTME Registry Collaborative. Dis Colon Rectum. 2019 Jul;62(7):794-801. Doi: 10.1097/DCR.0000000000001410- **Chapter 4**

Fulfilling the Portuguese Law, the Candidate declares to have actively participated in the collection and study of all material and data included in this Thesis, having written all papers. This Thesis and the work included in it have been approved by the Ethics Research Committee of NOVA Medical School and by Hospital Beatriz Ângelo Ethics Committee.

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Original paper 4 – “Transanal total mesorectal excision: 3-year oncological outcomes”

Original paper 5 - “Transanal versus laparoscopic total mesorectal excision. Comparative study of long-term outcomes”

Original paper 6 – “Oncological outcomes of local excision compared with radical surgery after neoadjuvant chemoradiotherapy for rectal cancer: a systematic review and meta-analysis”

Original paper 7 – “Loop ileostomy in rectal cancer surgery: factors predicting reversal and stoma related morbidity”

Collaborative paper 1 – “Incidence and Risk Factors for Anastomotic Failure in 1594 Patients Treated by Transanal Total Mesorectal Excision: Results from the International TaTME Registry”

Collaborative paper 2 – “Predictive Factors and Risk Model for Positive Circumferential Resection Margin Rate After Transanal Total Mesorectal Excision in 2653 Patients with Rectal Cancer”

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Acknowledgements

My interest in Colorectal Surgery began during my Surgical Residency while working with a colleague specially dedicated to this area. With this experience and throughout my professional development, I realised the importance of specialization to obtain true competence and skills in any medical area. The concept of “General” Surgery became, in my view, too generic and impossible to guarantee the high scientific standard I defend and that we all aim to attain.

My way of perceiving Surgery changed and I searched for specialization through internationalization at St Marks hospital, in London. Acknowledging it as the european reference centre for colorectal disease, as a resident I applied to a 6-month Laparoscopic Fellowship in that institution. This initial experience was decisive to make me embrace colorectal surgery. Upon returning to Portugal and finishing my residency, in 2011, I was invited by Professor Sue Clark to become a part of St Marks Hospital colorectal surgical staff for one year as a Resident Surgical Officer and Colorectal Consultant.

St Marks Hospital has always been a world reference in the treatment of colorectal disease. It has a remarkable history in the colorectal surgery evolution and is dedicated exclusively to this pathology with patients referred from all over the world. This hospital has an extraordinary clinical staff, which includes all the subspecialties involved in the treatment of the colorectal patient. I had the opportunity to work with extraordinary personalities of colorectal surgery such as John Nicholls, Robin Phillips, John Northover, Carolynne Vaisey, Robin Kennedy, Janindra Warusavitarne, Ian Jenkins and Sue Clark, in the context of clinical, investigational and academic excellency. I worked in oncology, pelvic floor, hereditary, inflammatory bowel disease and intestinal failure and it gave me exposure to a very high volume of complex pathology. St Marks hospital is also very dedicated to academic and research work and is involved in a multitude of projects from clinical trials to investigator-initiated, with regular publications in the most relevant international journals and contributing to the education of foreign doctors. The opportunity to experience how research was conducted and witness the interaction between investigators and clinicians was very stimulating and mind opening. I started working with Professor Sue Clark in Ileoanal pouch dysfunction and with Professor Carolynne Vaisey in colorectal disorders during pregnancy, which lead to the publication of several papers not included in this Thesis. This incredible setting was decisive to foster and blossom my interest in research. Recognizing the impact of this English journey in my professional life, I am forever in debt to everyone who I learned from in St Marks Hospital. To this day, Prof Sue Clark, Dr Ian Jenkins and Dr Janindra Warusavitarne remain my guides and friends.

Returning from St Marks, I was invited by Professor Rui Maio to embrace the opening of a new hospital in Lisbon, Hospital Beatriz Ângelo, as a dedicated colorectal surgeon. The construction of a Colorectal Unit with specialised and multidisciplinary medical staff was a very challenging but very gratifying experience, for which I am very thankful and proud. Since the foundation of our Hospital in 2012, I have been committed exclusively to colorectal surgery and particularly dedicated to rectal cancer and inflammatory bowel disease. While in Hospital Beatriz Ângelo, I had the opportunity of working with fantastic colleagues from Surgery, Oncology, Radiology and Gastroenterology with whom a solid colorectal team was created.

The complexity of rectal cancer and the daily difficulties of therapeutic decisions, in a constant search for balance between treatment-related benefits and morbidity, has always been a source of interrogations in my mind. This was the main drive to conduct the research presented in this Thesis. Although it shows only the results of the studies we conducted, for me it represents the pathway I decided to embrace, the search for answers, with scientific honesty and dedication to the patient. Demanding but rewarding.

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Last but not least, I want to thank my friends and family for always encouraging me throughout the difficulties of pursuing a research objective in parallel to a demanding surgical career. To Camila, for your belief in me.

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My final word is for my husband Rui, who always embraced me and kept me going. Nothing of this would have been possible without his love, friendship and support.

For Medicine and for my patients

Thesis Outline

This Thesis is composed of 8 chapters as outlined below. The structure of this Thesis reflects the research work featured in 7 original published pieces.

Chapter 1 presents the rational that lead to the work presented in this Thesis and the general and specific hypothesis within the scope of this work.

Chapter 2 approaches the landscape of rectal cancer, the central player of this thesis. This chapter brings into question the most recent perspectives in the diagnosis and treatment of this complex disease.

Chapter 3 analyses biological determinants in the treatment of rectal cancer specifically microRNAs as molecular predictors of response to neoadjuvant chemoradiotherapy.

Chapter 4 analyses surgical determinants in the treatment of rectal cancer namely the short and long-term clinical and oncological outcomes of a new surgical option, transanal total mesorectal excision. This chapter also approaches the controversies of local and radical resections following neoadjuvant chemoradiotherapy. Finally, it investigates the morbidity associated with loop ileostomy in rectal cancer surgery, studying predictive factors of reversal and stoma related morbidity.

Chapter 5 discusses the transversal findings and clinical implications of this work in the management of rectal cancer patients.

Chapter 6 encloses ongoing additional research derived from the works presented in this Thesis, namely on the impact of metabolism-related hormones on colorectal carcinogenesis, on rectal patient-derived organoids, on the watch and wait strategy after complete clinical response to neoadjuvant therapy and on total neoadjuvant therapy with intensification treatment.

Chapter 7 collects the original published papers that are the basis of this work.

Sumário

O cancro do recto é uma doença muito complexa que tem vindo a aumentar nas idades mais jovens com um enorme impacto na qualidade de vida. Esta é uma patologia extremamente heterogénea no que concerne ao seu comportamento, dependente de vários factores que determinam não só o seu curso mas a resposta à terapêutica.

Nas últimas décadas progressos significativos têm sido feitos na abordagem do cancro do recto devido a um melhor conhecimento da fisiopatologia da doença, conduzindo ao aparecimento de novas opções de tratamento. De forma síncrona com uma evolução técnica, o conceito terapêutico também se alterou, mudando de uma perspectiva exclusivamente focada nos *outcomes* oncológicos para um modelo com preocupações relacionadas com os resultados funcionais e a qualidade de vida. O ênfase passou também a residir na minimização dos efeitos deletérios do tratamento. Esta é a interrogação na base deste trabalho: **é possível encontrar determinantes biológicos e cirúrgicos do tratamento do cancro do recto por forma a diminuir a morbilidade associada à terapêutica mas obtendo igualmente os resultados pretendidos?**

Existem vários factores biológicos que influenciam os resultados terapêuticos do cancro do recto mas verifica-se, igualmente, um inquestionável impacto da opção cirúrgica selecionada. Sendo a nossa meta a obtenção dos melhores resultados com a menor morbilidade, é necessário procurar estes determinantes biológicos e cirúrgicos do tratamento óptimo.

O objectivo deste projeto é analisar possíveis determinantes da terapêutica do cancro do recto. São colocadas as seguintes questões: 1) poderemos optimizar a seleção dos doentes para quimioradioterapia neoadjuvante através da identificação de marcadores moleculares de resposta?, 2) poderemos melhor selecionar a técnica cirúrgica nomeadamente com a excisão total do mesorecto via transanal ou a excisão local em casos específicos? e 3) será possível uma melhor escolha dos doentes para ileostomia derivativa através da identificação de factores preditivos de morbilidade associada a este estoma?

A terapêutica neoadjuvante é atualmente administrada aos doentes com adenocarcinoma localmente avançado do recto, maioritariamente com boa resposta tumoral. Contudo, cerca de um terço dos doentes submetidos a quimioradioterapia não beneficiam deste tratamento, têm risco acrescido de progressão de doença durante o mesmo bem como de toxicidade desnecessária. Até hoje, não foram ainda validados quaisquer marcadores preditivos de resposta à quimioradioterapia que possam ajudar na seleção dos doentes para esta terapêutica. Tendo em conta o seu papel na oncogénese do cancro do recto bem como o seu envolvimento na resposta ao tratamento médico, colocámos a hipótese de os microRNAs em particular microRNA-16, microRNA-21, microRNA-135b, microRNA-145 e o microRNA-335 poderem ser biomarcadores de resposta à quimioradioterapia, predizendo os bons e os maus respondedores. Foi encontrada uma associação estatisticamente significativa entre a sobre-expressão de microRNA-21 no tecido tumoral pré- quimioradioterapia e pior resposta à mesma. Os nossos

resultados sugerem a possibilidade do microRNAs-21 ser um biomarcador de resposta patológica à quimioradioterapia no cancro do recto. A confirmação desta associação poderá ter uma translação para a prática clínica corrente, com a inclusão do miRNA nos algoritmos de decisão terapêutica, possibilitando uma melhor seleção dos candidatos a quimioradioterapia.

Durante os últimos 30 anos, grandes progressos cirúrgicos foram introduzidos no cancro do recto com vista à melhoria dos *outcomes* e diminuição da morbilidade associada ao tratamento. O mais recente avanço neste âmbito é a excisão total do mesorecto via transanal introduzida em 2010, com resultados a curto prazo muito positivos. Contudo, os *outcomes* a longo prazo são ainda controversos. Analisámos os outcomes oncológicos dos primeiros 50 doentes submetidos a esta técnica na nossa instituição e procedemos à sua comparação com os obtidos por um grupo equiparado de doentes submetidos a excisão total do mesorecto laparoscópica. Mesmo refletindo a curva de aprendizagem da nova técnica, foram encontrados valores semelhantes entre os grupos no que concerne a sobrevivência global, sobrevivência livre de doença e recidiva local a curto e longo prazo. Estes resultados apontam para que a excisão total do mesorecto via transanal possa produzir *outcomes* oncológicos seguros, compatíveis com o que tem sido publicado para a abordagem laparoscópica. Contudo, este estudo também enfatiza a sua exigente curva de aprendizagem e o risco significativo de morbilidade que lhe está associado. Na realidade, qualquer que seja a opção cirúrgica utilizada no tratamento do cancro do recto distal, é necessária elevada proficiência, sendo que resultados óptimos só se atingem com treino adequado e auditoria contínua como garante da sua melhoria à medida que a experiência aumenta.

Entendendo a excisão total do mesorecto como um dos grandes avanços no tratamento do cancro do recto, não podemos deixar de reconhecer o seu impacto negativo na qualidade de vida dos doentes com tumores distais. Neste contexto começaram a ser ponderadas estratégias terapêuticas menos agressivas com vista a uma menor morbilidade, nomeadamente quimioradioterapia seguida de excisão local. Através de uma revisão sistemática com metanálise que comparou, em contexto de neoadjuvância, os outcomes da excisão local com os da cirurgia radical, encontrámos valores de recidiva local, sobrevivência global e livre de doença semelhantes entre os grupos. Estes resultados podem ser explicados pelo facto de o mais importante determinante oncológico não ser o estadiamento inicial mas sim o pós quimioradioterapia, refletindo o comportamento biológico do tumor. No entanto, alguns estudos incluídos nesta metanálise apenas mostraram o estadiamento inicial. Na realidade, após a quimioradioterapia, a excisão local parece ser uma alternativa nos doentes com tumor restrito à submucosa e sem adenopatias objectiváveis (ycT1N0), nos doentes com co-morbilidades ou que recusam cirurgia radical.

Na cirurgia de excisão total do mesorecto é frequentemente realizada ileostomia derivativa por forma a reduzir as consequências do *leak* anastomótico. Contudo, a maioria dos doentes não enfrenta esta complicação sendo desnecessariamente exposta à potencial morbilidade do estoma. De facto, o efeito protetor do estoma derivativo deve ser contrabalançado com a sua morbilidade, bastante relevante. Tendo investigado marcadores de complicações associadas à ileostomia, identificámos a *Diabetes Mellitus* e a morbilidade da cirurgia rectal índex como factores preditivos não só de maior morbilidade associada ao

estoma e ao seu encerramento bem como de menor encerramento. Assim, quando ponderamos a realização de uma ileostomia derivativa na cirurgia do recto, há que ter em conta a influência destes fatores preditivos de morbilidade. É essencial individualizar as decisões terapêuticas e adoptar uma abordagem mais seletiva no uso do estoma derivativo, especialmente nos doentes em que o risco do mesmo pode superar as potenciais vantagens.

Em suma, existem vários factores que influenciam a conduta terapêutica na abordagem do cancro do recto. Existem determinantes biológicos e cirúrgicos do tratamento desta doença que necessitam de ser estudados, com vista ao atingir dos melhores resultados com a menor morbilidade. O papel dos microRNAs na oncogénese é inquestionável como o é a influência de microRNAs específicos, nomeadamente o microRNA-21, na resposta à quimioradioterapia neoadjuvante. Igualmente, também é crítica a opção cirúrgica nos diferentes contextos clínicos. De facto, podemos individualizar as intervenções cirúrgicas através do uso seletivo da excisão total do mesorecto via transanal nos tumores distais ou da excisão local pós quimioradioterapia nos doentes de alto risco com boa resposta, confinada à submucosa. Igualmente, antes da realização de cirurgia de excisão radical, é imperativo optimizar o status geral do doente e controlar factores de risco modificáveis como a *Diabetes* Mellitus por forma a diminuir igualmente a morbilidade associada ao estoma de proteção.

Palavras-chave: cancro do recto, microRNAs, microRNAs 21, excisão total do mesorecto via transanal, excisão local, ileostomia.

Abstract

Rectal cancer (RC) is a very complex disease that has been increasing in younger patients, imposing a great impact in quality of life. It is an extremely heterogeneous pathology in what regards to behaviour, which is dependent of many factors that determine its course and response to treatment. In the past decades, significant progress has been made in the management of RC due to a better knowledge of disease pathophysiology and consequent development of new therapeutic options. Synchronously with the technical evolution, the concept of oncological treatment also changed, from a perspective exclusively focused on survival outcomes to a model involving concerns with functional results and quality of life. Emphasis changed to minimizing the deleterious effects of treatment. However, many rectal cancer patients are still submitted to medical therapies and surgical options without any benefit and that even add unjustified morbidity. This is the core question of this work: **can we find biological and surgical determinants of RC treatment in order to decrease its related morbidity while achieving the intended outcomes?**

There are biological factors that influence clinical results and there is an undeniable impact of the surgical options we select. As our goal is obtaining the best possible outcomes minimizing morbidity, we must search for the biological and surgical determinants guidelining the optimal treatment.

The aim of this project is to provide new insights to possible determinants of RC treatment. We ask the following questions: 1) can we better select patients for chemoradiotherapy through the identification of molecular predictors of response?, 2) can we individualize the surgical technique for each RC patient, using transanal total mesorectal excision or local excision in selected cases? and 3) can we improve assortment of patients for a derivative ileostomy identifying factors predictive of related morbidity?

Neoadjuvant therapy is currently given to the majority of locally advanced rectal cancer with a majority of good tumour response. However, one third of patients that undergo chemoradiotherapy do not profit from this option, are at increased risk of disease progression and even unnecessary toxicity. So far, there are no validated predictors of response to chemoradiotherapy to aid in deciding whether the patient should or not undergo this therapy, avoiding related morbidity. Considering their role in rectal cancer oncogenesis and involvement in the response to medical therapies, we hypothesized that microRNAs (miRNAs or miRs), in particular microRNA-16, microRNA-21, microRNA-135b, microRNA-145 and microRNA-335 are biomarkers of response to neoadjuvant CRT, predicting good and bad responders. We found a statistically significant association of microRNA-21 overexpression in pre- chemoradiotherapy rectal cancer tissue and worse response. Our results suggest that microRNA-21 may, indeed, be a biomarker of pathological response in rectal cancer. Confirmation as such could translate into clinical application through the inclusion of the levels of microRNA-21 in algorithms of treatment decision, certainly allowing a better selection of candidates for chemoradiotherapy.

During the last 30 years, great surgical progress was introduced in RC treatment aiming to improve outcomes and diminishing the morbidity associated with treatment. The most recent of theses attempts is transanal total mesorectal excision, developed in 2010, which yielded very positive short-term results.

However, long-term outcomes are still controversial and not clarified. We analysed the oncological outcomes of the learning curve of this technique at our institution and compared them to a matched cohort of patients submitted to the standard of care laparoscopic total mesorectal excision. Similar long-term results regarding local recurrence, overall survival and disease-free survival were found. These results point out to the fact that transanal total mesorectal excision can produce short and long-term oncological safe results, compatible to what has been published for the laparoscopic approach. However, this work also emphasized the demanding learning curve and significant risk for morbidity associated with this novel technique. The fact is that, whatever option is used to performed distal RC surgery, it requires advanced surgical skills and optimal results can only be achieved with adequate training and continuous evaluation of outcomes to ensure they improve as experience grows. Transanal total mesorectal excision does not intent to replace other established approaches to rectal surgery but to add new alternatives to address difficult cases.

As we understand TME as one of the greatest revolutions of rectal cancer treatment we also acknowledge its negative impact on the quality of life of patients with distal tumours. In this setting, less aggressive therapeutic strategies started to be discussed in order to decrease therapeutic morbidity, namely neoadjuvant chemoradiotherapy combined with local excision. Through a systematic review and meta-analysis that compared the outcomes of local excision and radical surgery in the post neoadjuvant setting, we found similar outcomes between groups in relation to local recurrence, overall survival and disease-free survival. These results are explained by the fact that the most relevant determinant of local recurrence and survival is not the baseline staging but the post chemoradiotherapy one, that reflects tumour biologic behaviour. However, some studies included in this metanalysis were based on initial staging. In sum, after CRT, patients with an incomplete response contained in the mucosa or submucosa with negative nodes (ycT1N0) may be an indication for LE. This strategy can also be considered in trial setting or as an option for patients refusing abdominoperineal resection or with significant comorbidity.

Still in rectal cancer surgery, defunctioning ileostomy is frequently constructed to reduce the poor consequences of a leak. However, the majority of patients does not face anastomotic breakdown and is unnecessarily exposed to stoma potential complications. In fact, stoma protective effect needs to be balanced against its morbidity, which is actually quite high. We identified *Diabetes Mellitus* and complications of the index rectal surgery as predictive of higher ileostomy morbidity and of closure-related problems as well as lower ileostomy reversal. So, when deciding over diverting a colorectal or coloanal anastomosis, the influence of these predictive factors must be taken into account. It is essential to individualize treatment decisions and adopt a more selective approach concerning the use of a defunctioning ileostomy, especially for patients in which the risks of having a stoma may offset potential advantages.

In summary, there are many factors influencing the proper therapeutic conduct to follow in the approach of rectal cancer. There are biological and surgical determinants of the treatment of this disease that need to be analysed, in order to achieve the best results with the lowest morbidity. The role of the

microRNA in oncogenic pathways is undeniable as is the influence of specific microRNA, namely miR-21, in the response to chemoradiotherapy. Likewise, the choice of particular surgical interventions in different clinical settings can be critical to obtain the appropriate outcomes. We can individualize surgical options through the selective use of transanal total mesorectal excision in distal tumours or local excision in high risk patients with very good response, confined to the submucosa, in post neoadjuvant treatment. Likewise, prior to performing radical surgery, it is imperative to optimize patients and control modifiable risk factors as diabetes mellitus in order to decrease stoma-related morbidity.

Keywords: rectal cancer, microRNAs, microRNAs-21, transanal total mesorectal excision, local, excision ileostomy.

Acronyms and Abbreviations

AJCC: American Joint Commission on Cancer

APC: *adenomatous polyposis coli*

APER: abdominoperineal resection

AR: anterior resection

BMI: body mass index

CAP: College of American Pathologists

Ca 19.9: carbohydrate antigen 19.9

cCR: clinical complete response

CEA: carcinoembryonic antigen

ChT: chemotherapy

CI: confidence interval

CRC: colorectal cancer

CRC-CSC: colorectal cancer stem cells

CRM: circumferential resection margin

CRP: C reactive protein

CRT: chemoradiotherapy

CSC: cancer stem cells

CT: computed tomography

DHFU: dihydrofluorouracil

dMMR: deficient mismatch repair

DFS: disease-free survival

DP: distant progression

DPFS: distant progression-free survival

DPD: dihydropyrimidine dehydrogenase

DNA: deoxyribonucleic acid

DRE: digital rectal examination

DWI: diffusion-weighted imaging

ECOG: Eastern Cooperative Oncology Group

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

ELAPE: extralevator abdomino-perineal excision

EMT: epithelial-to-mesenchymal transition

EMVI: extramural vascular invasion

ERAS: enhanced recovery after surgery

ERUS: endorectal ultrasound

FAP: familial adenomatous polyposis

5FU: 5-fluououracil
FFPE: formalin fixed paraffin embedded
FOXO1: Forkhead box O 1
GTP: guanosine-5'-triphosphate
GTPase: hydrolase enzyme that hydrolase GTP
HNPCC: hereditary non-polyposis colorectal cancer
IBD: inflammatory bowel disease
IGF-IR: insulin-like growth factor receptor
IMRT: intensity-modulated radiation therapy
IRS-1: insulin receptor substrate-1
ISR: intersphincteric resection
lapTME: laparoscopic total mesorectal excision
LCCRT: long course chemoradiotherapy
LAR: low anterior resection
LARC: locally advanced rectal cancer
LARS: low anterior resection syndrome
LE: local excision
LND: lateral node dissection
LN: lymph node
LVI: lymphovascular invasion
LR: local recurrence
MAPK: mitogen-activated protein kinases
mRNA: messenger RNA
miRNA: microRNA
miR-16: microRNA-16
miR-21: microRNA-21
miR-135b: microRNA-135b
miR-145: microRNA 145
miR-335: microRNA-335
MR: magnetic resonance
mrTRG: magnetic resonance tumour regression grade
MMR: mismatch repair
MSI: microsatellite instable/ instability
MSI-H: microsatellite unstable –high
MSS: microsatellite stable
MRF: mesorectal fascia
NCCN: National Comprehensive Cancer Network
NSAID: non-steroidal anti-inflammatory drugs
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
OD: odds ratio

OS: overall survival
pCR: pathological complete response
PDCD4: programmed cell death 4
PDO: patient-derived organoids
PET-CT: positron emission tomography–computed tomography
PNI: perineural invasion
Phosphatidylinositol-3-kinase-AK: PI3-K-AKT
pre-miRNA: precursor miRNA
pri-miRNAs: miRNA primary transcripts
PRISMA: preferred reporting items for systematic reviews and meta-analyses
pTRG: pathological tumour regression grade
PTEN: phosphatase and tensin homolog
QoL: quality of life
RC: rectal cancer
RCT: randomised controlled trial
RFS: recurrence-free survival
RISC: RNA-induced silencing complex
RMA: residual mucosal abnormalities
RNA: ribonucleic acid
ROC: receiver operating characteristic
RR: relative risk
RS: radical surgery
RT: radiotherapy
SCRT: short course radiotherapy
SSI: surgical site infection
siRNA: small interfering RNAs
SC: stem cells
SNAI1: snail Family transcriptional repressor 1
TAMIS: transanal minimally invasive surgery
TATA: transabdominal transanal
TaTME: transanal total mesorectal excision
TEMS: transanal endoscopic microsurgery
TP: thymidine phosphorylase
TME: total mesorectal excision
TNT: total neoadjuvant therapy
TRG: tumour regression grade
TS: thymidylate synthase
VEGFR1: vascular endothelial growth factor receptor 1
VEGFA: vascular endothelial growth factor A
WW: Watch and Wait

Foreword

Cancer is the disease of the century.

Cancer imposes an enormous global health burden, estimated to affect over 18 million of individuals worldwide, of whom 9.6 million die [1]. As global population grows, aging and lifestyle habits boost cancer, the disease burden continues to increase. The expected number of affected individuals will rise to 43.8 million over the next 20 years [1]. According to the latest statistics, lung, breast, colorectal, prostate and stomach are the top 5 most common cancer types, regardless of gender. Colorectal cancer (CRC) is the second deadliest overall [1] (Figure 1).

Cancer starts when normal cellular processes fail and a rogue cell originates a group of cells that share its abnormal capabilities or behaviours. If these cells are uncontrollably efficient they grow, surpass their usual boundaries, invade the contiguous body parts and spread to other organs.

Each type of cancer presents diverse molecular and phenotypic features in a very complex setting, which brings up different clinical challenges. Many questions are still raised regarding cancer behaviour, selection of patients for the different available therapeutic options, response to treatment and, very importantly, morbidity associated with it. Such is the case of Rectal Cancer (RC), to which this Thesis is dedicated.

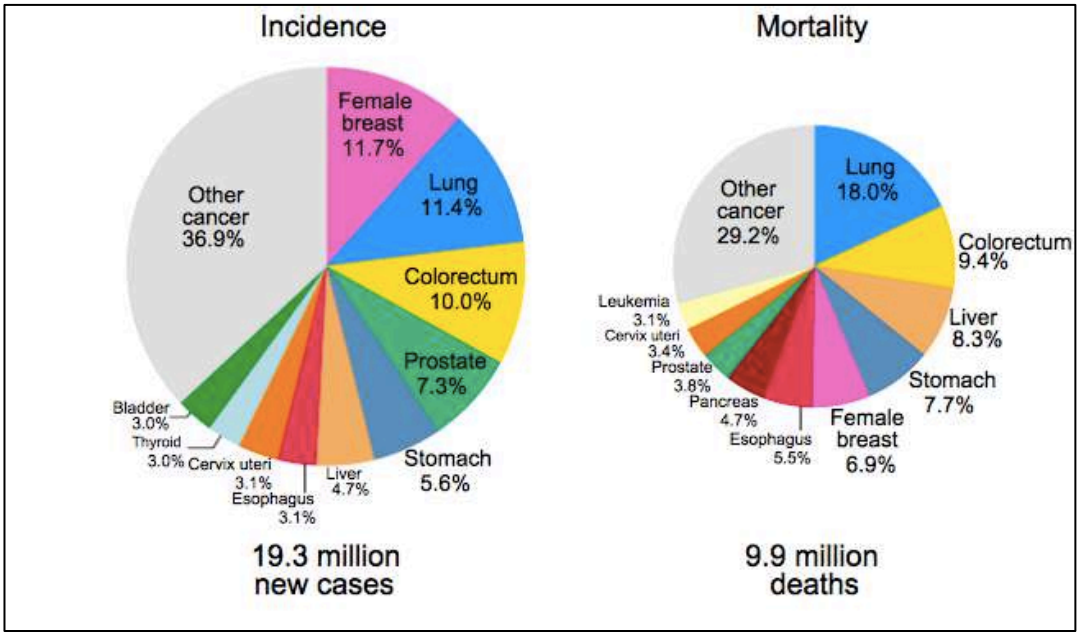

Figure 1 Distribution of cases and deaths for the top 10 most common cancers in 2020 for both sexes. Non-melanoma skin cancers are included in the "other" category. Reproduced from *Global Cancer Statistics 2020: GLOBOCAN estimates of Incidence and Mortality Worldwide for 36 cancers in 185 countries. CA CANCER J CLIN 2021; 0: 1*

Chapter 1

Rational for Research Presented in this Thesis and Hypothesis

As many other neoplasia, rectal cancer (RC) is currently a health problem not only for its high prevalence but also for how treatment impacts on patients.

Until the beginning of the XX century, RC was largely treated by perineal excision and sigmoid colostomy, an approach that lead to high rates of early local recurrence (LR), almost 100%, and poor disease-free survival (DFS). In 1908, Miles introduced the concept of oncological surgery based on the notion of lymphatic spread, according to location. This translated into a new surgical resection with abdominal and perianal approaches, abdominoperineal resection (APER), that gave sight to a major decrease in recurrence (down to 30%). With documented proof that this was a better surgical strategy, the use of the APER continued to grow over the first 4 decades of the 20th century. In parallel, the operative mortality declined due to overall improvements in anaesthesia, patient selection and operability.

In 1939, Claude Dixon of the Mayo Clinic introduced the anterior resection for cancers of the rectum and recto-sigmoid with performance of an anastomosis. Experience with sphincter preservation grew, particularly after surgeons returned to practice from World War II. During the XX century, many surgical advances came up, namely new techniques, instruments and staplers, allowing more distal anastomosis.

One of the greatest improvements in RC treatment came from the introduction of total mesorectal excision (TME) by Heald (2). The approach showed a relation between the removal of the mesorectal package and the decrease in locoregional recurrence. Subsequently, Quirke [3] exposed that this was also associated with the plane of surgical dissection and specimen quality. Indeed, LR was a consequence of inadequate resection with involvement of the circumferential resection margin (CRM). Demonstrating better oncologic outcomes with lower than 5-10% LR, TME became the standard surgical approach for treating RC [4] .

Synchronously with this “surgical evolution”, other medical strategies came to place in RC treatment. In the early 20th century, radiotherapy (RT) had its start in several centres in both Europe and United States and George Binkley introduced radon seed implants and radium therapy for RC patients. While he originally intended RT for nonsurgical candidates, some of the specimens from patients who went on to resection demonstrated involution of the primary tumour, prompting the idea of RT as a response inducing treatment, to be used in combination with surgery. Patients with RC usually underwent surgery alone, resulting in high rates of pelvic failure with subsequent morbidity and death. In the pre-TME era, most LR appeared within a field of the pelvis that could be encompassed by radiation portals. Radiotherapy became the logical adjuvant for “high-risk” patients. During the 80s and 90s, chemotherapy was added to RT proving a decrease in pelvic failure rates and an improved survival, leading to its incorporation as chemoradiotherapy (CRT) into the routine management of patients with resected stage II/III disease.

In the latest decades, the concept of oncological treatment also changed in RC. From a perspective exclusively focused on oncological outcomes, it gradually moved to a model involving concerns with functional results and quality of life. Emphasis was now on the importance of minimizing the deleterious effects of treatment.

Although producing extraordinary advances in RC treatment, this evolution pathway brought innumerous new interrogations, many still unanswered. We are increasingly evolving to a precision

Medicine with individualized treatment strategies to optimize oncological outcomes and, at the same time, decrease treatment-associated morbidity. Notwithstanding, many RC patients are still submitted to medical therapies and surgical options without any benefit and that even add unjustified morbidity. This is the interrogation on the basis of this work: **can we find biological and surgical determinants of RC treatment in order to decrease its related morbidity while achieving the intended results?**

Currently, a lot of controversy surrounds various aspects of RC. One of the most relevant issues in debate relates to neoadjuvant CRT. This therapy is currently given to the majority of locally advanced mid and low RC to achieve downstaging and complete response (cCR), increase R0 resections, allow sphincter-sparing surgery and decrease LR. The fact is that, after neoadjuvant treatment, almost 25% of patients have no residual tumour identified while in 45–60% there is downstaging, and up to 30% actually exhibit resistance to CRT [5]. Overall, one third of patients that undergo neoadjuvant CRT may not profit from it. These non-responders are at increased risk of disease progression during CRT and of unnecessary toxicity caused by it. Thus, pre-treatment prediction of good and bad responders could be key in deciding whether the patient should or not undergo neoadjuvant CRT, avoiding related morbidity.

The inclusion of molecular markers in the algorithm to select patients for CRT could potentially allow for a better assortment of candidates. No biomarkers are yet validated and RC patients are still treated based solely on clinical stage. Classically, TNM staging system represents the most relevant prognostic factor to guide the prediction of oncologic outcomes and treatment recommendations. However, staging systems have reached their limit of usefulness encouraging the assimilation of other clinical, pathological and molecular parameters. In this setting, it is known that colorectal carcinogenic pathways and cellular response to oncological therapies are influenced by microRNAs (miRNAs or miRs), namely miR-16, miR-21, miR-135b, miR-145 and miR-335.

Considering their role in oncogenesis, **we hypothesized that these miRNAs are biomarkers of response to neoadjuvant CRT, predicting good and bad responders**.

The impact of the surgical strategy in patients' outcomes is undeniable and we evolved from one single technique performed in all RC patients to a multitude of procedures, individually selected according to patient performance status, oncological risk or even response to neoadjuvant therapies. In fact, during the last 30 years, great surgical progresses applied to RC treatment, namely laparoscopy and robotics, with the objective of improving outcomes and diminishing the morbidity associated with treating this condition. Despite this evolution, RC radical surgery is still associated with high rates of anastomotic leak, low anterior resection syndrome (LARS), incomplete TME specimens and conversion, with the associated worst prognosis [6]-[9]. Obese male patients with narrow pelvis and bulky distal tumours are technically very demanding due to restricted visibility, limited working space and difficulties in distal stapling. Transanal TME (TaTME) was introduced in 2010 to try to overcome these difficulties [10]. With very positive short-term results, the scientific community received this technique with enthusiasm. However, this reverse

proctectomy presented particular challenges associated with the change in anatomic perspective and the demands of a single-port technique, bringing new unusual morbidity, namely CO2 embolism and urethral lesions. Also, there are inconsistencies regarding oncological outcomes, as they are reported by most authors with short follow-up time, not allowing definitive conclusions do be drawn.

With the perception of TaTME as a new surgical possibility to address more difficult cases and with only known short-term results, we raised the following questions: **what are the mid and long-term outcomes of TaTME performed for RC? Are they comparable to the ones of standard of care laparoscopic TME?**

TME is, indeed, a major surgical procedure with significant morbidity that includes anastomotic leak, nerve injury, bowel, sexual and bladder dysfunction, that might require a temporary or permanent stoma [8],[9]. These circumstances brought to light another important question, the need to reduce the morbidity associated with TME, eventually with less aggressive therapeutic strategies.

Neoadjuvant therapy followed by organ-preserving procedures, like local excision (LE) or the Watch and Wait (WW) approach, started to emerge. As neoadjuvant therapy leads to significant improvements in local disease control, transanal full-thickness LE could be been considered for the management of selected patients with significant response to CRT.

In this setting, we aimed to review all the available literature on LE performed after CRT and analyse its most controversial aspects, namely the tumour scatter, the completion and salvage surgeries as well as LE- related morbidity. Also, we intended to **analyse if the outcomes of CRT followed by LE approach could be comparable to the ones of radical TME following CRT.**

In rectal surgery, a defunctioning ileostomy is frequently constructed to decrease morbidity and mortality associated with dehiscence of colorectal or coloanal anastomosis. However, this protective effect needs to be balanced against stoma morbidity. In fact, overall ileostomy morbidity is reported as high as 35% and relates not just to the management of the stoma itself but to the reversal procedure. Loop ileostomy complications include skin problems, leakage from the stoma dressing, retraction, prolapse, parastomal hernia, dehydration and electrolyte disturbance from high output [11]-[13]. Also, stoma reversal has a high overall complication rate, postoperative ileus and surgical site infection (SSI) being the most common. Moreover, having an ileostomy significantly impacts on the quality of life and a relevant part of the so-called “transient” stomas are never reversed.

In this setting, we hypothesized that ileostomy complications could be predicted allowing individualized decisions on endorsing or avoiding diversion and its morbidity. We raised the following questions: **can we, in the pre-treatment setting, identify factors that predict complications of stoma management, reversal and transformation into a permanent one?**

Considering the aforementioned knows and unknowns, this Thesis was dedicated to explore the **Hypothesis** that **there are biological and surgical determinants in RC treatment and that morbidity can be decreased in various ways**, namely by:

1) Better selecting patients for CRT through the identification of molecular predictors of response
2) Better selecting the surgical technique for each RC patient, namely TaTME or LE
3) Better selecting patients for a derivative ileostomy identifying factors predictive of stoma morbidity

In this work, we have addressed the following specific aims:

- To explore the recent advances and interrogations in RC approach, namely in staging, surgical techniques and neoadjuvant therapy. Chapter 2, Original Paper 1

- To investigate the association between miR-16, miR-21, miR-135b, miR-145 and miR-335 expression in rectal non-neoplastic and tumour tissue and response to neoadjuvant CRT and oncological outcomes. Chapter 3, Original Paper 2

- To investigate the association between circulating miR-21 and response to neoadjuvant CRT and oncological outcomes. Chapter 3, Original Paper 3

- To investigate the mid-term clinical, pathological and oncological outcomes of TaTME, in order to selectively apply this technique in RC patients. Chapter 4, Original Paper 4

- To investigate the long-term clinical, pathological and oncological outcomes of TaTME in RC and compare them with the standard of care technique, laparoscopic TME. Chapter 4, Original Paper 5

- To compare the oncological outcomes of 2 different surgical strategies following neoadjuvant CRT, namely local excision (LE) and radical surgery. Chapter 4, Original Paper 6

- To analyse the morbidity related to diverting ileostomy in RC surgery, identify predictive factors of complications related to stoma management, reversal and conversion into a permanent one, as well as the impact of anastomotic techniques on morbidity. Chapter 4, Original Paper 7

Chapter 2

New Perspectives in Rectal Cancer

Original Paper 1

Management of Rectal cancer: Times are Changing

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ABSTRACT

Approximately one third of all colorectal malignancies are located in the rectum. It has long been recognized that rectal cancers behave differently from colonic tumours, namely in terms of local recurrence. For this reason, specific protocols have been developed to manage this disease both in staging procedures as well as in neoadjuvant and adjuvant treatments. Magnetic resonance imaging is now obligatory for rectal cancer staging. Also, preoperative chemoradiotherapy is recommended in the majority of locally advanced rectal with obvious advantages in downstaging and downsizing tumours, sometimes allowing sphincter-sparing procedures. Total mesorectum excision is the rule when operating on rectal cancer. Despite these advances, there are still unanswered questions, namely the utility of neoadjuvant protocols in low lying, early stage tumours with the aim of performing a local excision and the guidance of restaging of disease after neo-adjuvant treatment. In fact, evaluation of response to therapy became a cornerstone of individualized rectal cancer treatment. Finally, there is the concern that with current protocols we are overtreating some patients that would not need such extensive treatment. In this review, we critically examine recent advances and controversies in staging, surgery, and chemoradiotherapy in the management of patients with rectal cancer.

Keywords: rectal cancer, neoadjuvant therapy, restaging, surgery

RATIONAL AND AIMS

Rational: Despite the great advances in rectal cancer approach, many unanswered questions remain. Based on a review paper co-written by the PhD Candidate, this article serves as an introduction to Rectal Cancer, to which this Thesis is dedicated.

Aims: The present appraisal aimed to summarize the state of the art as well as the most relevant controversial issues in rectal cancer approach, namely in staging, surgical techniques and neoadjuvant therapy.

INTRODUCTION

Rectal malignancies comprise lesions less than 15 cm from the anal verge, as measured by rigid proctoscopy, tumours being classified as low (up to 5cm from the anal verge), middle (from 5 to 10cm) or high (from 10 up to 15 cm).

Rectal cancer (RC) encompasses approximately 25% of all primary colorectal cancers (CRC) and follows a different natural disease course than colonic tumours, with distinctive complications and recurrence patterns. This lead to the establishment of specific protocols for RC, particularly the use of magnetic resonance (MR) imaging for staging and the use of neoadjuvant therapies for selected cases. Advances in the management of patients with RC in the last decade contributed to a marked improvement in patients' outcomes. In the United States 5-year survival increased from 49.2% in the 70s' to 68.5% in the 2000-2005 period with a similar trend observed in Europe [14],[15].This benefit may be related to disease detection at an earlier stage and widespread use of optimal surgery with TME but also to a multidisciplinary approach in specialized centres with an increased use of both radiotherapy and chemotherapy, ideally in a neoadjuvant context [15],[16].

Despite advances, the heterogeneity and unpredictability of RC biological behaviour and response to treatment makes it still a very defying disease.

Epidemiology and risk factors

In 2020, more than 1.9 million new colorectal cases and 935 000 deaths were estimated to have occurred worldwide, representing one in 10 cancer cases and deaths. Last year colorectal cancer ranked third in terms of incidence and second in terms of mortality [1]. Rectal cancer (RC) corresponded to 35% of all colorectal malignancies, imposing a global health burden accounting for the considerable incidence and mortality. In the European Union there were 125 000 cases with a median age at diagnosis of 70 years and an annual mortality of 4-10/100 000 population [17] .

Over the last decades, a gradually decline in RC incidence and mortality rates have been globally observed, thought to be a result of earlier diagnoses through screening, healthier lifestyle choices (e.g. declines in smoking) and better treatment modalities. Interestingly, data show that the decline is among those aged 65 years or older but there is an increase among individuals younger than 65 years, with a 1% annual rise in those aged 50 to 64 years and 2% annual growth in those younger than 50 years [1]. In fact, it is predictable that the incidence of colon and rectal cancers will increase by 90.0% and 124.2%, respectively, for patients 20 to 34 years by 2030. The cause of this trend is currently unknown but CRC that occurs in young adults may be clinicopathologically and genetically different from the one in older adults.

On the other hand, as CRC is considered a marker of socio-economic development, there is a geographic impact on incidence, and RC rates have been steadily rising in specific areas namely Eastern Europe, Eastern and South-Central Asia and South America. Incidence is highest in Eastern Asia and tends to be low in most regions of Africa. Portugal is also a high incidence area. The increase in formerly lower human development index countries likely reflects changes in lifestyle factors, namely dietary and social (Fig.1).

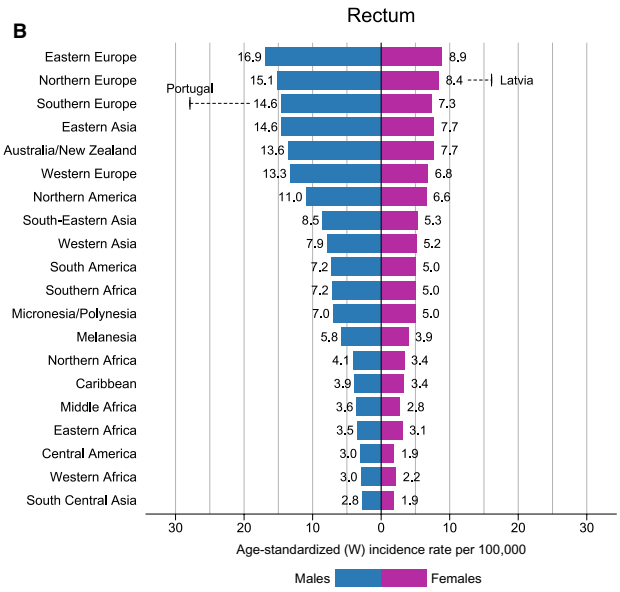

Figure 1 Region-specific incidence age-standardized rates by sex for rectal cancer in 2020. Reproduced from *Global Cancer Statistics 2020: GLOBOCAN estimates of Incidence and Mortality Worldwide for 36 cancers in 185 countries. CA CANCER J CLIN 2021; 0: 1-41*

It is well recognized that individuals with inflammatory bowel disease (i.e. ulcerative colitis and Crohn's disease) are at an increased risk for CRC [18]. Possible risk factors for the development of CRC include smoking, consumption of red and processed meats, moderate/ heavy alcohol use, *diabetes mellitus*, low levels of physical activity, high body mass index (BMI)/ obesity and metabolic syndrome [19],[20]. Approximately 20% of cases are associated with familial clustering and there is an increase risk of CRC in first-degree relatives of patients with colorectal adenomas or invasive CRC [21],[22]. Genetic susceptibility includes well-defined inherited syndromes, such as Lynch syndrome (hereditary non-polyposis colorectal cancer [HNPCC]) and familial adenomatous polyposis (FAP) [23],[24].

On the contrary, healthy lifestyle, exercise, regular uses of vitamin D, aspirin or non-steroidal anti-inflammatory drugs (NSAID) seem to decrease the risk for CRC [25],[26]. Finally, the relationship between diabetes and CRC is complex [27]. Whereas diabetes and insulin use may increase the risk of developing CRC, treatment with metformin appears to decrease it, at least in women [27],[28].

Staging, risk assessment and preoperative optimization

Determining the optimal treatment plan for an individual patient with RC is a complex process. Preoperative staging has two main objectives: to define the treatment intent (curative or palliative) and determine therapeutic options and prognosis.

Staging process begins with digital rectal examination (DRE) but the accuracy of assessment ranges from 58% to 88%, largely depending on the surgeon's experience [29]. The precise localization of tumours, especially those beyond the reach of an examining finger, mandates for rigid proctoscopy, considered as the single most useful tool.

Imaging plays a critical role in staging, both for evaluating the primary tumour and to assess for the presence of distant metastases. Pelvic magnetic resonance (MR) is the most accurate technique to define locoregional staging with proven high sensitivity and specificity in the estimation of T and N stages and in the prediction of mesorectal fascia (MRF) and circumferential resection margin (CRM) involvement prior to surgery [29]-[38]. The presence of >1 mm between tumour or involved node and MRF, levator muscles or intersphincteric plane defines a clear CRM. In contrast, an involved or threatened CRM is the one with tumour/ node within 1 mm of MRF or levator muscle. High-quality MR allows further sub classification of cT3 into T3a -T3d. By detecting extramural vascular invasion (EMVI), T stage and CRM status, MR can also predict the risks of LR and synchronous/ metachronous distant metastases [7],[40] (Fig 2 and Fig 3).

Endorectal ultrasonography (ERUS) can be used in RC staging when MR is contraindicated (e.g. presence of a pacemaker) or in superficial T1 lesions, although low-lying, very high or near-obstructive

tumours are major drawbacks to the use of this technique. MR should be used in all other RCs. Furthermore, ERUS cannot fully image bulky rectal tumours nor regions beyond the immediate area of the primary tumour (i.e. tumour deposits, vascular invasion, MRF), being highly operator dependent.

For systemic staging (Table 1), chest imaging should be by performed with computed tomography (CT) scan, whereas imaging of the abdomen can be performed with CT or MR. Synchronous lung metastases occur in approximately 4% to 9% of patients with RC [41]-[43] and studies have shown that 20% to 34% of patients with RC present with liver metastases [44]. Positron emission tomography–computed tomography (PET-CT) imaging cannot be recommended routinely and should only be used to evaluate equivocal findings on a contrast-enhanced CT scan, in patients with a high risk of metastases (i.e. extensive EMVI on MR) or in patients with a strong contraindication to intravenous contrast (Table 1) [45],[46].

Complete work-up also includes total colonoscopy (to evaluate synchronous lesions), histopathologic analysis of the specimen obtained via biopsy or local excision (i.e. excised polyps) with mismatch repair (MMR) or microsatellite instability (MSI) testing, determination of carcinoembryonic antigen (CEA) and assessment of performance status (Eastern Cooperative Oncology Group – ECOG) for operative risk. Also, consideration must be given to the likely functional results of treatment, including the probability of maintaining or restoring normal bowel function/ anal continence, preservation of genitourinary function and fertility.

Perioperative optimization through Enhanced Recovery After Surgery (ERAS) protocols have sustained improved short-term and oncological outcomes [47]. In Hospital Beatriz Ângelo, ERAS protocol was implemented in 2017 for all elective colorectal surgery including RC surgery. This protocol relies on the perioperative patient optimization in a multidisciplinary setting including surgery, anaesthesia, nutrition, nursing, stoma care, imunohemotherapy, physiotherapy and pneumology. If removal of the rectum is contemplated, early consultation with an enterostomal therapist is recommended for preoperative marking of stoma site and patient teaching purposes.

In patients with distal RC, in particular, the simultaneous achievement of the goals of cure and of minimal impact on quality of life can be challenging.

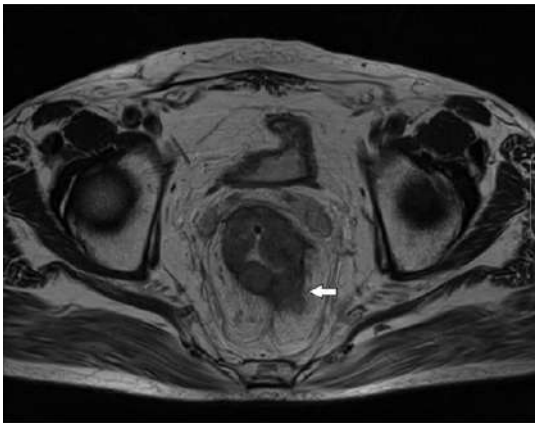

Figure 2 Axial T2-weighted MR images. Distance between rectal tumour and MRF is less than 1mm (white arrow) representing a threatened MRF. Reproduced from *Cravo M, Rodrigues T, Ourô S et al. Management of rectal cancer. Times are changing. GE Port J Gastroenterol. 2014.*

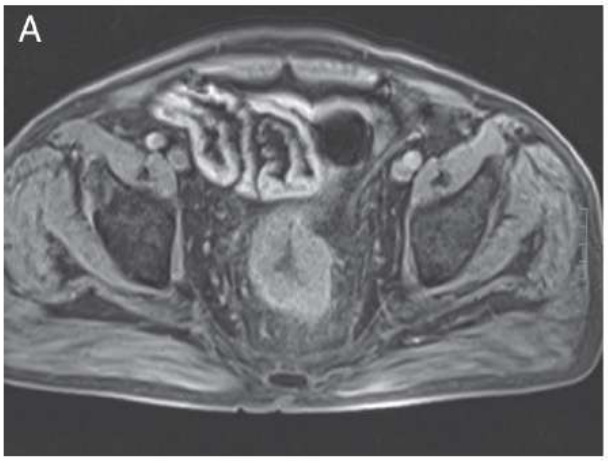

Figure 3 (A) High definition axial T1-weighted MRI post-gadolineum clearly depicts rectal tumour with transmural stranding in mesorectal fat. Reproduced from *Cravo M, Rodrigues T, Ourô S et al. Management of rectal cancer. Times are changing. GE Port J Gastroenterol. 2014*

Table 1 Diagnostic work-up in primary RC

Parameter	Method
Location	DRE
	Rigid/ flexible protoscopy
Histological analysis	Biopsy
cT Stage	
- Early	ERUS, MR
- Intermediate/ advanced	MR, (ERUS*)
Sphincter infiltration	MR (ERUS*, DRE*)
cN Stage	MR, (CT, ERUS*)
cM Stage	CT, MR of the liver and abdomen
	CT of the thorax
	PET-CT if extensive EMVI for other sites
Evaluation for all patients	MDT

CT, computed tomography; DRE, digital rectal examination; EMVI, extramural vascular invasion; ERUS, endorectal ultrasound; MDT, multidisciplinary team; MR, magnetic resonance; PET, positron emission tomography; * Less optimal methods.

TNM Staging

RC is staged according to National Comprehensive Cancer Network (NCCN) Guidelines – American Joint Commission on Cancer (AJCC) Cancer Staging system, 8th edition. Classically, TNM staging system is the most important tumour related prognostic factor that guides treatment recommendations and prediction of oncologic outcomes (Table 2 and Table 3). However, this staging system has reached its limit of efficacy making other clinical, pathological and molecular parameters relevant.

Table 2 TNM RC staging according to NCCN Guidelines, AJCC Cancer Staging system 8th edition

TNM Staging	
T- Primary tumour	Tx – primary tumour cannot be accessed T0 – no evidence of primary tumour Tis – carcinoma in situ: invasion of lamina propria T1- tumour invades submucosa T2- tumour invades muscularis propria T3- tumour invades subserosa or into non-peritonealized perirectal tissues T4- tumour directly invades other organs or structures and/ or perforates visceral peritoneum T4a - tumours directly penetrate visceral peritoneum T4b - tumours directly invade other organs or structures
N- Regional lymph node	Nx – regional lymph nodes cannot be accessed N0- no regional lymph nodes metastasis N1- metastasis in 1–3 regional lymph nodes N1a- metastasis in 1 regional lymph node N1b- metastasis in 2–3 regional lymph nodes N1c- tumour deposit(s) in the subserosa, mesentery, or non-peritonealized pericolic or perirectal tissues without regional nodal metastasis (i.e., satellite tumour nodules) N2- metastasis in 4 or more regional lymph nodes N2a- metastasis in 4-6 regional lymph nodes N2b- metastasis in 7 or more regional lymph nodes
M- Metastatic disease	M1- Distant metastasis M1a: Metastases confined to one site/ organ (lung, liver, ovary, non-regional lymph nodes, without peritoneal metastasis) M1b: Metastases in more than one site/ organ M1c: Metastasis to the peritoneum with or without organ involvement

Table 3 TNM RC staging according to NCCN Guidelines, AJCC Cancer Staging system 8th edition

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage IIIA	T1-T2	N1/ N1c	M0
	T1	N2a	M0
Stage IIIB	T3-T4a	N1/ N1c	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
Stag IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b
Stage IVC	Any T	Any N	M1c

Within each T stage, survival is inversely correlated with N stage. T stage has more prognostic value than N stage so patients with stage IIIA disease (T1–2N+) have longer cancer-specific survival than patients with stage IIA (T3N0), IIB (T4aN0) and IIC (T4bN0) [48].

Not included in TNM classification, other variables defined by pelvic MR are considered for staging, namely EMVI and CRM status, an independent risk factor of local recurrence (LR) [40]. In fact, the Mercury study [40] was published with the aim of assessing the prognostic relevance of CMR predicted by high resolution MR. Five-year results of this trial showed that MR accurately assessed CRM preoperatively, differentiating patients with high and low risk. Patients with MR-clear CRM had a 5-year overall survival (OS) of 62.2% compared with 42.2% in those with involved CRM (Hazard Ratio (HR) 1.97; 95% Confidence Interval (CI), 1.27–3.04; $p < 0.01$). The preoperative MR imaging also predicted DFS (HR 1.65; 95% CI, 1.01–2.69; $p < 0.05$) and LR (HR 3.50; 95% CI, 1.53–8.00; $p < 0.05$).

In this setting, treatment protocols that include preoperative radiotherapy should consider these findings. The assessment of the relationship between tumour and CRM is more important to decision-making than lymph node (LN) status. In fact, CRM is superior to AJCC TNM-based criteria for assessing local, distant recurrence and OS [40].

Post-treatment staging / Re-staging

Reassessment of response to neoadjuvant therapy is done with 2 objectives: 1) to plan the surgical approach for achievement of a clear CRM and 2) to identify response grade and modify treatment strategy accordingly, particularly in complete responders [49].

Restaging relies on digital rectal examination (DRE), proctoscopy and re-imaging through high definition pelvic MR with functional techniques (dynamic contrast-enhanced diffusion-weighted imaging – DWI), that provides valuable prognostic information and distinguishes viable tumour from fibrosis or inflammatory from neoplastic LN (Fig 4) [49]-[54].

CRT causes tumour necrosis, which is then replaced by inflammatory tissue and fibrosis. High definition MR allows the measurement of microcirculation, vascular permeability, tissue cellularity, however, post CRT restaging is a challenge due to these radiation-induced changes. The degree of response is classified according to magnetic resonance Tumour Regression Grade (mrTRG) that can discriminate between good/ bad responders and predict survival outcomes (Table 4)[56]. There is, still, inter-reader variability in this evaluation and an incomplete correlation of radiological TRG (mrTRG) with histopathological TRG [57]. Nevertheless, high definition MR has been shown to accurately distinguish patients with post treatment tumours confined to the *muscularis propria* or more superficially from more advanced ones [57].

Routine restaging of chest and abdomen after neoadjuvant CRT is not recommended, but patients with cT4 cancers, threatened CRM and the presence of EMVI should be re-staged within 3 months of original staging to exclude metastatic disease prior to surgery [48].

Table 4 Magnetic Resonance Tumour Regression Grade (mrTRG)

Magnetic Resonance Tumour Regression Grade (mrTRG)
mrTRG 1 - Complete radiological response (linear scar only)
mrTRG 2 - Good Response (dense fibrosis, no obvious tumour signal)
mrTRG 3 - Moderate response (> 50% fibrosis and visible, intermediate signal)
mrTRG 4 - Slight response (mostly tumour)
mrTRG 5 - No response/ regrowth of tumour

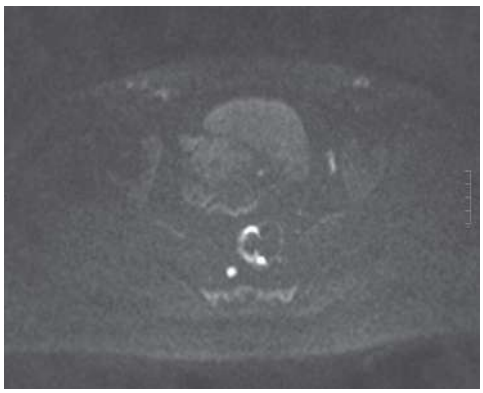

Figure 4 After CRT, axial diffusion-weighted shows hyperintensity of the node and rectal wall involved by tumour. Reproduced from *Cravo M, Rodrigues T, Ourô S et al (2017). Management of rectal cancer. Times are changing. GE Port J Gastroenterol. 2014*

Histopathology

Early tumours resected endoscopically or through local excision (LE) should be taken *en bloc* without piecemiel to assess invasion of resection margin and deepest area. For mesorectal resections, histopathological examination should include the evaluation of the total mesorectal excision (TME) quality, based on completeness of the mesorectum and surgical excision plane. Importantly, specimen quality impacts on survival and LR [7].

A TME specimen ideally should have a smooth surface without incisions, defects or cracks, as an indication of successful surgical excision of all mesorectal tissue. 'Coning' represents the tendency for the surgeon to cut towards the central tube of the rectum during distal dissection rather than staying outside the visceral mesorectal fascia. The specimen that shows a tapered, conical appearance represents suboptimal surgical quality [3].

Along with the CRM involvement, TME quality represents a surrogate parameter for oncological outcomes and 3 different planes of surgery were defined, mesorectal, intramesorectal and *muscularis propria* planes (Table 5 and Fig 5) [6].

More advanced T-stage, tumour distance from the anal verge less than 8 cm, higher age and low surgical case volume have been independently associated with moderate or poor TME quality [6] [58]. In the radical specimen extramural vascular invasion (EMVI), lymphovascular invasion (LVI), perineural invasion (PNI) and tumour budding should also be evaluated.

Table 5 TME plane of surgery

Mesorectal plane (good plane of surgery)	Intact mesorectum with only minor irregularities of a smooth mesorectal surface; no defect deeper than 5 mm; no coning; and smooth circumferential resection margin on slicing
Intramesorectal plane (moderate plane of surgery)	Moderate bulk to mesorectum with irregularities of the mesorectal surface; moderate distal coning; *muscularis propria* not visible with the exception of levator insertion; and moderate irregularities of CRM
***Muscularis propria* plane** (poor plane of surgery)	Little bulk to mesorectum with defects down onto *muscularis propria;* very irregular CRM; or both

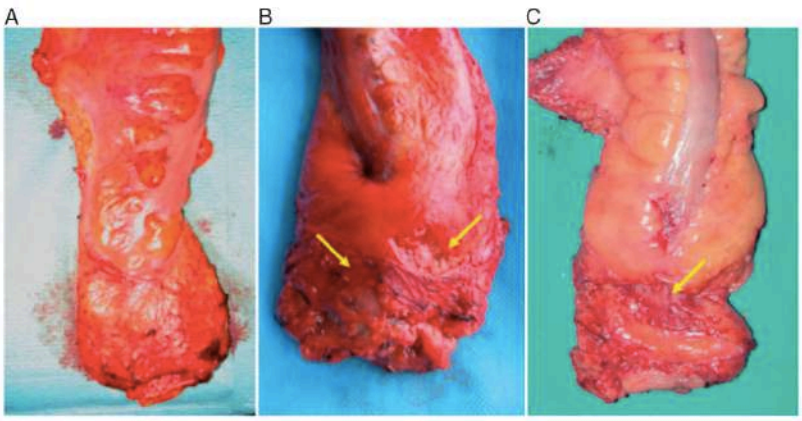

Figure 5 Definitions of quality of mesorectal excision (A) A complete mesorectal excision defines good bulk of mesorectum with a smooth surface and no defects, (B) A nearly complete mesorectal excision shows good bulk of mesorectum, but some defects or irregularities in the surface (arrowed) are present and (C) An incomplete mesorectal excision demonstrates a deep defect on the mesorectum below the peritoneal reflection, which allows visualisation of the *muscularis propria* (arrowed). Reproduced from *R. Glynne-Jones et al on behalf of the ESMO Guidelines Committee (2017). ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology 28 (Supplement 4): iv22–iv40*

Parameters influencing prognosis

There are postoperative histopatological features that influence prognosis and impact on LR such as pathological TNM stage, T substage, CRM status, number/ proportion of involved lymph nodes (LN), extracapsular extension, extranodal tumour deposits, tumour differentiation, lymphovascular invasion (LVI), extramural vascular invasion (EMVI), perineural invasion (PNI) and pathological tumour regression grade (pTRG) [6].

Circumferential Resection Margin status

The CRM is the closest radial margin between the deepest penetration of the tumour or LN and the edge of resected tissue around the rectum, measured in millimetres (mm). Accurate pathologic assessment of the CRM is crucial because it is a strong predictor of both LR and OS, even in patients with neoadjuvant therapy [3]. Positive intranodal CRM is associated with lower LR rates than a positive CRM by direct tumour extension [3].

Lymph Nodes

The AJCC and the College of American Pathologists (CAP) recommend evaluation of 12 lymph nodes (LN) to accurately stage RC [48],[59]. The mean number of LN retrieved after neoadjuvant therapy is significantly less than by surgery alone (13 vs. 19, $p < 0.05$; 7 vs. 10, $P \leq 0.0001$) however this can be a marker of a higher tumour response and better prognosis [60]-[62]. Although in the past histologically involved nodes have been associated with a high risk of LR, this risk is decreased with good quality mesorectal excision, ensuring removal of all mesorectal ganglia.

Pathological Response to Treatment

Chemoradiotherapy (CRT) causes tumour necrosis, which is then replaced by inflammatory tissue and ultimately fibrosis. Pathologists can quantify the ratio of viable tumour cells to fibrosis to generate a pathological tumour regression grade (pTRG) [63]. Using neoadjuvant CRT protocols, expected rates of pathologic response range from 5% to 42% and this is associated with low rates of local and distant recurrence.

There are various classifications of pTRG, the most consensual being the one according to the CAP that grades pTRG on a scale of 0 (complete response – no viable cancer cells observed) to 3 (poor response – minimal or no tumour kill; extensive residual cancer). Inter-observer agreement is limited and response is also classified differently by other classifications (e.g. pathological complete response (pCR) is classified by Mandard as TRG1 but by Dworak as TRG4) [63],[64] . The optimal system, however, remains nuclear (Table 6).

Table 6 Pathological Tumour Regression Grade (pTRG) classifications

CAP	Mandard	Becker	Dworak	Rödel
0.Complete response (no viable tumour cells)	1.Complete regression (fibrosis without detectable tumour)	1a. No residual tumour/ tumour bed	0. No regression	0. No regression
1. Near complete response (Single cells or rare small groups of cancer cells)	2. Fibrosis with scattered tumour cells	1b. <10%residual tumour/ tumour bed	1. Predominantly tumour with significant fibrosis and/or vasculopathy	1.Regression of <25% of tumour
2. Partial response (no viable tumour cells (Residual cancer with evident tumour regression, but more than single cells or rare small groups of cancer cells)	3. Fibrosis and tumour cells with preponderance of fibrosis	2.10-50%residual tumour/ tumour bed	2. Predominantly fibrosis with scattered tumour cells (slightly recognizable histologically)	2.Regression of <25-50% of tumour
3. Poor or no response (Extensive residual cancer with no evident tumour regression)	4. Fibrosis and tumour cells with preponderance of tumour cells	3. >50%residual tumour/ tumour bed	3. Only scattered tumour cells in the space of fibrosis with/without acellular mucin	3. Regression of >50% tumour
	5. Tissue of tumour without changes of regression		4. No vital tumour cells detectable	4. Complete regression

Perineural Invasion

Several studies have demonstrated that the presence of perineural invasion (PNI) is associated with a significantly worse prognosis. There is a 4-fold greater 5-year survival in patients without PNI in comparison to patients whose tumours invade nearby neural structures. Multivariate analysis of patients with stage II RC showed that patients with PNI have a significantly worse 5-year DFS compared to those without PNI (29% vs. 82%; p = 0.0005)[65]. Outcomes were analogous in stage III disease [66]. A meta-analysis that included almost 23000 patients in 58 studies found that PNI is associated with a worse 5-year DFS (Relative risk (RR) 2.35; 95% CI 1.66–3.31) and 5-year OS (RR 2.09; 95% CI 1.68–2.61)[67]. PNI is therefore considered a high-risk factor for distant recurrence.

Extranodal Tumour Deposits

Extranodal tumour deposits or pN1c are irregular discrete tumour deposits in the perirectal fat away from the edge of the primary tumour, within its lymphatic drainage but without lymph node tissue. Not considered as LN replaced by tumour, they are thought to be due to LVI or occasionally PNI. Tumour deposits are associated with decrease DFS and OS [65],[66] and patients with pN0 had a 91.5% 5-year survival rate compared to 37.0% for patients with pN1c tumours ($p < 0.0001$) [70]. Another retrospective study found a similar difference in 5-year OS rates (80.3% vs. 34.9%, respectively; $p<0.001$) [71]. This association with decreased survival also happens in patients with neoadjuvant CRT [69].

Extramural vascular invasion

Extra-mural venous invasion (EMVI) is the presence of malignant cells within an endothelial cell–lined space that either is surrounded by a rim of smooth muscle or contains erythrocytes [72].

The morphologic features of EMVI on baseline T2-weighted MR range from discrete serpiginous or tubular projections into the perirectal fat following the course of a visible vessel to, in more advanced cases, the vessel being expanded by tumour [73]. EMVI is a very important predictor of disease-free survival (DFS), with a 3-year DFS rate of 35% for patients with EMVI in comparison with 74% for those with without this 48 feature [17],[71].

Likewise, the degree of pathologic EMVI influences the likelihood of nodal dissemination, liver metastasis and survival rates [72].

Surgical options

The main aim of surgical treatment of RC is to reduce the risk of residual disease and local relapse while preserving sphincteric, urinary and sexual functions. There are a variety of surgical options, which depend not only on tumour location and stage but also on patient sphincter function. These options include local procedures such as classic transanal local excision, transanal endoscopic microsurgery (TEM) or transanal minimally invasive surgery (TAMIS) and radical procedures involving TME, with or without an anastomosis, performed by open, laparoscopic or robot-assisted approach, transanally or transabdominally.

Local excision

Local excision (LE) procedures are reserved for selected cases with a low likelihood of nodal metastasis, dependent on T stage, differentiation and LVI. For tumours staged as T1, associated LN metastasis have been reported in 6-11% of patients while T2 cancers have a 10-20% risk of nodal involvement, this risk increasing to 33-58% in T3 tumours [74]. The incidence of nodal metastases also relates to tumour differentiation with up to 50% of poorly differentiated tumours exhibiting positive nodes [74]. Transanal LE is, therefore, only appropriate for selected small T1, well to moderately differentiated, mobile tumours and with no evidence of nodal involvement, LVI or PNI.

In the post neoadjuvant setting LE is more controversial. The ACOSOG trial Z604129, a single-arm study evaluating the oncologic outcome of patients with cT2N0M0 distal RC treated with CRT followed by LE, showed this as an organ-preserving alternative in patients within this stage who refuse, or are not candidates, for transabdominal resection [75]. Moreover, the observation that complete mucosal response often corresponds to negative LNs [48],[75] supports the idea of less aggressive surgical treatments in patients submitted to CRT with good response. What is not known is if a LE in the post neoadjuvant context would be appropriate for patient other than high-risk ones.

Local therapies are appealing because of their technical ease, low complication rate, rapid post operative recovery with minimal mortality and morbidity (sphincter-sparing procedure) and, above all, because they avoid the need for a permanent stoma in early, distally located RC [77]. The major drawback to local procedures include the absence of nodal pathological staging, mainly because there is evidence that LN micrometastases also exist in early RC and are unlikely to be identified by imaging. In fact, patients undergoing LE have higher LR rate and positive margins than those undergoing radical resection. In T1N0 patients, a small but significant decrease in OS was also noted in the LE group [77]-[79].

If unfavourable features are observed on pathological examination (high grade, positive or indeterminate margins, PNI, LVI or invasion into the lower third of the submucosa - sm3 level) a radical excision is warranted [81].

Parks transanal LE is appropriate for small lesions located in the 8 cm distal rectum. Transanal endoscopic microsurgery (TEM) is a minimally invasive surgical technique originally described by Buess *et al* in the 80s' [77],[82], which uses a transanal approach with a set of endoscopic surgical instruments and a form of enhanced or assisted vision. TEM facilitates excision of more proximal lesions reaching until 20 cm from the anal verge. Transanal minimally invasive surgery (TAMIS) is a more recent surgical approach that utilizes single-port platforms and laparoscopic instruments with the creation of a pneumorectum. TAMIS involves a shorter platform than TEM with wider working angle that allows more distal and easier dissection (Fig. 6).

Whatever surgical option used, 'Parks excision', TAMIS or TEM, all LE techniques require a full thickness excision performed perpendicularly through the bowel wall with a deep margin outside the *muscularis propria* into the mesorectal fat and a mucosal margin with 1 cm or more around the target lesion [81]. However, anatomic considerations may prevent LE even if tumour staging is appropriate. In large lesions, full thickness excision with or without primary closure can lead to loss of rectal volume and stenosis, with poor functional results, specially if post pelvic radiation.

A recent meta-analysis found that TEM and TAMIS achieve superior oncologic outcomes compared with transanal Parks LE due to less fragmentation and inferior positive margins, despite having a demanding learning curve.

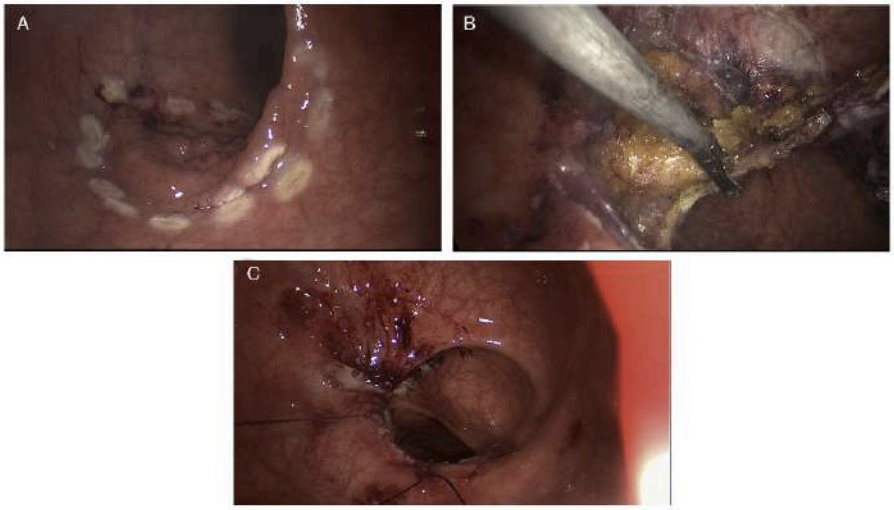

Figure 6 TAMIS resection of a cT1N0 at 20 cm from the anal verge (A) Delimitation of tumour margins (B) Full thickness excision performed perpendicularly through the bowel wall into the perirectal fat (C) Final closure of the operative wound. Reproduced from *Cravo M, Rodrigues T, Ourô S et al. Management of rectal cancer. Times are changing. GE Port J Gastroenterol. 201*

Radical resection

Patients with RC who do not meet requirements for LE should be treated with a radical resection with sphincter preservation, if oncologically safe.

In the late 1970s, Heald *et al* developed the technique of total mesorectal excision (TME) demonstrating that, in some cases, nests of tumour cells outside LN could be found in the mesorectum and would be left behind by a ‘‘conventional’’ anterior resection [2]. Using TME alone, the author achieved LR rates of less than 5% and emphasis became focused on the CRM [3],[82]-[84]. Over the last three decades, TME has brought a dramatic improvement in the outcome of surgery for RC [4].

The TME approach is designed to radically remove the lymphatic drainage of tumours located above the level of the levator muscles and involves an *en bloc* excision of the mesorectum, associated vascular, lymphatic structures, fatty tissue and MRF as a “tumour package” through sharp dissection sparing the autonomic nerves. The lymphatic drainage regions of rectal tumours are influenced by their position in the rectum. Distal tumours have both upward and lateral drainage, whereas proximal ones are more likely to have only upward mesorectal drainage [86]. So, for more proximal tumours there should be a 5 cm oncological margin from the distal end of the tumour but 1 cm margin is acceptable for very distal ones, especially after neoadjuvant CRT, thereby allowing sphincter-sparing procedures.

Currently, lymphadenectomy is perceived differently among countries. In Japan, lateral node dissection is practiced if the tumour is sited below the peritoneal reflection, to reduce the risk of pelvic recurrence and improve OS. In Europe, extension of nodal dissection beyond the classic field of resection is only recommended if lateral nodes are clinically suspicious, enlarged or persisting following CRT.

In cases where anal function and distal clearance are adequate, anterior resection (AR) with TME may be followed by creation of a colorectal or coloanal anastomosis. Limitations for sphincter-sparing procedures are beginning to be regarded as mostly functional and not just oncological [87],[88]. In patients with very distal tumours not usually considered for a sphincter-sparing surgery, intersphincteric resection (ISR) is indicated. Involvement of the internal sphincter is not a contraindication but ISR should not be performed in fixed tumours involving the external sphincter or levators neither in patients with poor preoperative continence [89],[85].

When resection with safe margin carries the loss of continence (direct involvement of the sphincter or levators) or when preoperative continence function is already compromised, an abdominoperineal resection (APER) is indicated. This technique involves en bloc resection of the rectosigmoid, the rectum and mesorectum (TME), anus and perianal soft tissue with the need to create a colostomy. Although it has been the gold standard treatment of distal RC, APER it is nowadays performed in less than 5% of all cases [91].

Retrospective comparative studies revealed that APER has higher LR and reduced survival than AR [16],[90]. This difference in outcome may be explained by the fact that tumours below the peritoneal reflection are usually at an upper stage, have a lymphatic drainage which might not be included in the TME and are at higher risk of lateral pelvic LN involvement. Also, in the distal third of the rectum the mesorectum disappears at the top of the sphincter so, below this level, the sphincter itself constitutes the CRM. Distal tumours have a shorter distance to cross until they reach the CRM as compared to more proximal tumours, “protected” by a thicker mesorectum. Based on the study of the morphometry of the surgical specimen,

West *et al* [89],[93] demonstrated that APER specimens have less tissue volume around the tumour when compared with AR, and are associated with a greater CRM involvement, LR and less OS. Also, there appears to be an association between the APER procedure itself and the increased risks of recurrence and death, related to a technically demanding procedure.

This problem could be overcome with a ‘‘new’’ APER, introduced by Holm *et al* [94], more cylindrical and closer to the original Miles description, with removal of additional tissue around the tumour, reducing the probability of CRM involvement [88],[93]. This extralevator APER (ELAPE) involves wider perineal dissection, in prone position, with removal of the anal canal, levators and coccyx from below, closing the perineal defect with flaps. ELAPE may have benefits over a conventional APER, including lower rates of intraoperative perforation, CRM involvement and LR, although inconsistencies are seen between studies [93],[94].

Laparoscopic approach

Despite the universally acceptance of the laparoscopic approach to the colon cancer treatment, the extension to RC remained, until recently, inexplicably controversial. Some groups still expressed oncological concerns based on the first results of the CLASICC trial [97] that compared laparoscopic to open resection, reporting higher rates of CRM involvement and a trend for worst male sexual function in the laparoscopic group. These results were not reproduced with longer follow-up and no significant differences were observed in 5-year LR, DFS or OS between groups, thereby encouraging the use of laparoscopic approach in RC [95]-[98] . With the same methodology, the COREAN trial randomized patients with stage II or III low- to mid RC to an open or laparoscopic resection and demonstrated no differences in 3- year DFS, 72.5% (95% CI, 65.0- 78.6) for open surgery and 79.2% (95% CI, 72.3-84.6) for the laparoscopic group.

The phase III COLOR II trial [8], powered for non-inferiority, randomized patients with localized RC to laparoscopic or open surgery. Patients in the laparoscopic arm had inferior blood lost, shorter hospital stays and a quicker return of bowel function although longer operative time. No significant differences were seen in completeness of resection, positive CRM, morbidity or mortality, neither 3-year LR, DFS nor OS.

Two other trials, ACOSOG Z6051 [9] and ALaCaRT [99], reported no differences between laparoscopic and open surgery regarding CRM involvement, distal margin and TME completeness, 2-year DFS, locoregional and distant recurrence. The criteria for non-inferiority of the laparoscopic approach were not met in the initial results but the techniques were found not to differ in oncological outcomes after longer follow-up. These outcomes are still corroborated by the results from National cancer databases and meta-analyses that consistently found laparoscopic approach to be safe and feasible [100]-[104]. A meta-analysis published in 2017 found, however, that the risk for a non-complete mesorectal excision was significantly higher in patients receiving a laparoscopic resection [107].

In conclusion, the majority of studies have shown that laparoscopy is associated with similar short- and long-term outcomes when compared to open surgery, however, others have shown higher rates of CRM positivity and incomplete TME. In this setting the minimally invasive resection of RC should only be performed by experienced surgeons, in colorectal dedicated units [108] .Laparoscopic TME

(lapTME) has a demanding learning curve and a meticulous technique is required. An international group of experts has defined standards for the technical details of lapTME. During the time working at St Marks Hospital, the PhD candidate produced an educational video contemplating laparoscopic low anterior resection. This video intended the step-by-step-learning, based on real patient surgical images and complementary anatomic drawings of the technique (https://www.stmarksacademicinstitute.org.uk/resources/laparoscopic-high-anterior-resection/).

Robotic approach

Several studies have compared the outcomes of robotic-assisted resection to conventional laparoscopic resection. Comparable results are generally seen between both approaches in relation to conversion to open resection, TME quality, postoperative complications and quality of life. Despite the ergonomic superiority of the technique, so far a significant benefit of robotic-assisted over laparoscopic surgery for RC has not been proven [107]-[110].

Transanal Total Mesorectal Excision

RC surgery can be very challenging especially in the particularly defying group of obese male patients with bulky distal tumours. This comes as a consequence of the difficulty of pelvic dissection related to limited operative field, decreased mobilization and stapling. Despite being a major technical leap, laparoscopic TME (lapTME) is associated with high conversion, anastomotic leak, suboptimal specimen, LARS, sexual and urinary problems [8],[111]. In this context, transanal total mesorectal excision (TaTME) was introduced to overcome technical difficulties in pelvic approach. This reverse proctectomy (down-to-up) performed with laparoscopic instruments through the anal canal was developed by Sylla and Lacy in 2010 [10].

TaTME is performed in a two teams approach (Cecil approach) with an abdominal team performing laparoscopic anterior resection working synchronously with a perineal team approaching the rectum from below, both in the mesorectal plane. This technique has several potential advantages with superior dissection of anterior tumours, improved visualization of nerve bundles and pelvic floor muscles and better determination of an appropriate distal margin. Also, it potentially improves dissection in the male narrow pelvis, decreases conversion and improves specimen quality, not requiring the difficult technical step of stapling distal to the tumour [114]. However, TaTME has specific problems associated with the change in anatomic perspective and the demands of single-port technique, with new complications unusual in laparoscopic and open approaches, particularly carbon dioxide (CO_2) embolism and urethral injuries [115]. Also, although short-term outcomes appear similar or better than standard laparoscopic resection [116]-[120], there are still unknowns regarding oncological outcomes and long-term results.

Chemoradiation treatment

The absence of a serosa surrounding the rectum, its close proximity to pelvic structures and technical difficulties associated with obtaining clear surgical margins carries a risk of locoregional recurrence. Neoadjuvant therapy for stage II or III RC, either chemoradiotherapy (CRT) or short-course radiotherapy (SCRT), includes locoregional treatment to minimize that risk.

For patients with locally advanced rectal cancer (LARC), treatment decisions regarding neoadjuvant therapy should be based on preoperative MR prediction of CRM, EMVI and more advanced T3 substages (T3c/T3d), defining the risk of LR and synchronous/ metachronous metastatic disease [59].

For resectable cancers, when there is no indication on MR that surgery is likely to be associated with threatened or invaded CRM and consequent R2 or R1 resection, standard TME should achieve a curative resection [48]. Combined-modality CRT plus surgery plus chemotherapy (ChT) is recommended for the majority of patients with stage II or stage III and several therapy sequences have been proposed for clinical practice. Also, in patients with pre-treatment stage I disease (T2N0), neoadjuvant CRT therapy may be considered in distal tumours with the aim of downsizing, increasing the chances of a sphincter sparing procedure and a clinical complete response (cCR) for a non-operative approach [121]. However, patients with lower risk of LR (i.e. proximal cT3, N0, M0, clear CRM with favourable prognostic features) may be adequately treated with surgery without CRT.

Previous studies have consistently shown that postoperative 5-fluorouracil (5-FU)-based CRT significantly improved local control and survival compared with surgery alone [122] . When radiotherapy was compared to CRT given pre-operatively, the German Rectal Cancer Trial [123] confirmed that the latest had a significant decrease in acute and late toxicities concomitantly with a better local control and higher chance of sphincter preservation. Since then, the standard treatment for locally advanced, clinically resectable (T3 and/or N+) distal RC is preoperative CRT. The total duration of perioperative therapy, including CRT and ChT, should not exceed 6 months.

Strategies of Pre-operative Radiotherapy

Advances in radiation physics and computer technology made possible to perform more precise radiation. Conformational radiotherapy uses CT images to map the location of a cancer in 3 dimensions. The two main strategies of preoperative radiotherapy are long-course chemoradiation (LCCRT) and short-course radiotherapy (SCRT).

1. LCCRT is recommended when CRM and/or R0 resection status are predicted to be at risk and implies the delivery to the pelvis of 25 to 28 fractions of 1.8-2 Gy over 5-6 weeks, with a total dose of 45-50.4Gy, using three or four fields. A boost with a further 5.4 Gy in 3 fractions can be considered if the CRM is threatened. RT fields should include the tumour or tumour bed with a 2 to 5 cm margin, the mesorectum, the pre-sacral and the internal iliac nodes. The external iliac nodes should also be included for T4 tumours involving anterior structures, as should inguinal nodes for tumours invading the distal anal canal. Positioning and other techniques to minimize radiation to the small bowel are encouraged. LCCRT also involves the administration of concurrent 5-FU or capecitabine and is the most accepted approach worldwide [124] . The

addition of chemotherapy to RT is done to potentiate local radiotherapy sensitization, to control micrometastases, induce tumour downsizing and/or downstaging and increase pathological complete response (pCR). In a study [125] of patients with T3–4 M0 randomly assigned to receive either preoperative RT alone or preoperative CRT with 5-FU/ leucovorin (LV), no difference in OS or sphincter preservation was observed in the two groups, although patients receiving CRT were significantly more likely to exhibit a pCR (11.4% vs. 3.6%; p <0.05), grade 3/4 toxicities (14.6% vs. 2.7%; p< 0.05) and less likely to have LR (8.1% vs. 16.5%; p <0.05). The addition of 5-FU/ LV enhanced the tumoricidal effect of RT with significant reductions in tumour size, pTN stage, lymphatic invasion, EMVI and PNI rates [116],[117] with no effect on OS, 30-day mortality, sphincter preservation and late toxicity [128],[129].

Although 5-FU continuous infusion is the conventional regimen used [122], two studies showed that capecitabine has similar rates of pCR, sphincter-sparing surgery and toxicity [124]. Similarly, capecitabine was non-inferior to 5-FU with regard to 5-year OS (capecitabine 75.7% vs. 5-FU 66.6%; p = 0.0004), showing an improvement in 3-year DFS (75.2% vs. 66.6%; p =0.034) [111],[114]. Capecitabine is equivalent to 5-FU in perioperative CRT and is an acceptable alternative to infusional 5-FU in patients able to manage the responsibilities inherent in self-administered, oral chemotherapy [120]. So, both agents can be used in the neo-adjuvant setting. Regarding the addition of oxaliplatin there are contradictory results and lack of a clear long-term survival benefit, while it increased toxicity with more adverse events [130]-[132]. The addition of bevacizumab, cetuximab, panitumumab or irinotecan to RT is currently also not recommended.

2. The second strategy of preoperative radiotherapy, traditionally used in Scandinavia, consists of SCRT that delivers a total dose of 25 Gy over 5 days in 5 fractions, without ChT and followed by immediate surgery (less than 10 days from the first radiation fraction) [133]. The rationale for SCRT regimen is that the short time period for delivery of the dose may neutralize the effects of accelerated cellular repopulation, a phenomenon characteristic of tumour cells exposed to radiotherapy. SCRT with delayed surgery is also a useful alternative to conventional SCRT with immediate surgery, offering similar oncological outcomes and lower postoperative complications [134].

It is not possible to provide a rigid definition of which T and N sub-stages require SCRT or LCCRT. The selection of preoperative approach is based on the ressecability and risk of a positive CRM. If CRM and/ or R0 resection *status* are predicted at risk, LCCRT is advised, particularly for patients with distal tumours. Otherwise, either SCRT or LCCRT can be administered. Rectal cancers above the peritoneal reflection do not seem to benefit from preoperative SCRT or LCCRT and should be treated as colon cancer. Patients with higher cT4 tumours falling into the pelvis might benefit from neoadjuvant treatment.

In patients with cT3/4 RC, LCCRT is associated with a relative 50% risk reduction in LR whereas SCRT does not result in apparent downstaging of tumours in terms of nodal status. Two large randomized controlled trials (RCT) studied the effect of SCRT in both LR and 5-year survival [133],[133] . Although the results of both these trials favoured SCRT, both were performed before the widespread introduction of TME surgery and, therefore, it remains to be proven whether this beneficial effect would had been observed if TME had been performed. Overall, both preoperative SCRT and LCCRT reduce the rate of LR in mid/low stage II/III RC without improvement of OS and with significantly worse intestinal and sexual functions after surgery.

Delayed surgery after radiotherapy

Tumour response to CRT is a time-dependent phenomenon and the optimal interval between CRT and surgery has not been clearly defined. This interval requires a balance between allowing sufficient time for the maximal effects of the RT to be fully expressed before tumour repopulation and the settling of acute reaction so that surgery can be carried out safely. It is also known that patients with “near complete” response can evolve to cCR, if given time [49].

The Lyon R90-01 study compared a period of less than 2 weeks with 6-8 weeks and found improved T and N downshift with longer intervals [135]. In a review from Cleveland Clinic, there was a steep increase in pCR after 7 weeks, which reached a plateau only after 12 weeks [136]. Although longer intervals from completion of CRT to surgery have been shown to be associated with an increase in pCR rates [137]–[139] it is unclear whether such longer intervals are associated with clinical benefit. Results of one national cancer database analysis suggested that an interval of more than 8 weeks was associated with increased odds of pCR whereas other similar analyses concluded that an interval over 56 or 60 days (8–8.5 weeks) was associated with higher rates of positive margins, lower rates of sphincter preservation and/or shorter survival [140]. The GRECCAR6 phase III multicentre RCT [141] randomized patients with stage II/III RC treated with CRT to a 7-week or an 11-week interval before surgery. The rates of pCR, anastomotic leak and the mean length of hospital stay (LOS) did not differ between groups (15.0% vs. 17.4%; p = 0.60), but the morbidity (44.5% vs. 32%; p = 0.04), medical complications (32.8% vs. 19.2%; p =0.01) and rate of complete mesorectal resection (78.7% vs. 90%; p = 0.02) were worse in the 11-week group.

Longer intervals after SCRT or LCCRT may enhance pCR rates with unknown prognostic implications but apparently without decline in survival [142],[143]. Consolidation ChT has been defended by recent studies to overcome the risk of repopulation and subsequent metastases, and an interval of 7 weeks but less than 12 weeks is now recommended for initial post radiotherapy restaging.

Change of surgical strategy based on post-treatment staging

Restaging after neoadjuvant CRT identifies responders in who planned treatment based on the original presentation might no longer be indicated. The post-treatment assessment often shows tumour downsizing and T or N downshifting, enabling sphincter preservation or even no surgical resection [49]. Observations strongly support the hypothesis that there is a close relationship between post-treatment T stage and risk of persistent LN metastasis. For this reason, Habr Gama *et al* consider that mucosal response can be viewed as a proxy for LN response [49],[144].

There is indeed a change in surgical strategy based on post CRT staging. In the German Rectal Cancer Study Group pre-treatment surgical recommendation was compared with the surgical procedure

after neoadjuvant CRT and 40% of patients originally thought to need APER actually underwent a sphincter-preserving procedure, without oncologic compromise at a median follow-up of 45 months [123].

The level of response to neoadjuvant treatment not only changes surgical strategy but also correlates with prognosis, LR, long-term oncological outcomes and distant metastasis [143],[144]. In the MERCURY trial [35], mrTRG was significantly associated with OS and DFS and patients with poor mrTRG had lower 5-year OS and DFS than the ones with good mrTRG (OS 27% versus 72%, p = 0.001 and DFS 31% versus 64%, p = 0.007). Similarly, in the CAO/ARO/AIO-94 trial [147], patients with clinical complete response (cCR) had a 10-year cumulative incidence of distant metastasis of 10.5% and DFS of 89.5% while those with poor regression had incidences of 39.6% and 63%, respectively. In a retrospective review of 725 patients, 5-year recurrence-free survival (RFS) rates were 90.5%, 78.7%, and 58.5% for patients with complete, intermediate, and poor responses, respectively ($p < 0.001$) [148].

In the post treatment restaging, degree of response classifies patients as good or bad responders according to mrTRG. Patients with mrTRG 1-2 are classified as good responders and those with mrTRG 3-5 as poor ones (Table 4)[149]. There is, however, inter-reader variability and incomplete correlation of mrTRG with histopathological TRG. High-definition MR and an experienced radiologist are crucial for this response evaluation hence dictating the treatment pathway to follow.

Watch-and-Wait Non-operative Approach for Clinical Complete Responders

Following neoadjuvant therapy 50-60% of patients are downstaged and 10-40% show a clinical complete response (cCR) [150]. The problem is that cCR has only partial concordance with pathological complete response (pCR), meaning that not all patients with complete clinical response are indeed total pathological responders nor all patients restaged as having an incomplete response will have malignant cells in the protectomy specimen.

TME carries high morbidity with a 2-8% mortality rate, rising to 30% in patients over 85 years. In this setting, a central questions arises: in the presence of a cCR after CRT, knowing that TME is associated with significant morbidity and mortality, is radical surgery and its possible complications justified only for the sake of confirming a pCR?

With this argument, some authors proposed a new algorithm in which therapeutical approach is based on response to neoadjuvant treatment [49]. The strategy incorporates response to CRT in treatment planning and sets the stage for considering less radical operative options or a “Watch and Wait “ approach.

This “Watch and Wait” (WW) tactic was introduced in 1998 by Habr Gama *et al* [151] paralleling for RC what had been happening for anal cancer. This conduct is a no-immediate surgical approach, recommended only in highly selected patients with cCR with intensive follow-up through digital, endoscopic examinations and pelvic MR, especially during the first year. Although not universally agreed, criteria for cCR have been defined as the absence of any palpable tumour or irregularity at DRE, no visible lesion at rectoscopy except a flat scar, telangiectasia or whitening of the mucosa and the absence of residual tumour on MR in the primary site or draining nodes. Also, an initially raised CEA level returning to normal (< 5ng/ml) after CRT is associated with an increased likelihood of cCR and pCR [152].

Acknowledging response to neoadjuvant therapy as a time-dependent event, the concept of "near-complete" response also emerged, defining patients with very good but incomplete response that can eventually evolve to cCR, if given time [153]. In fact, patient restaging is essential to decide for conservative or surgical strategies. If initially staged as good responders, patients can undergo a second period of "waiting" to allow more downstaging and eventual achievement of complete response.

Habr Gama *et al* retrospectively compared the outcomes of 71 patients who were observed without surgery following cCR (27% of patients) to the outcome of 22 patients (8%) who had incomplete clinical response but pCR post-TME. There were no differences between groups concerning survival outcomes. In the non-operative group 5-year OS and DFS were 100% and 92%, respectively, compared to 88% and 83% in the resected group [151]. These authors republished their series several times with longer follow-ups, reproducing the same excellent oncogical results [152]-[156].

A recent prospective study [159] included a more thorough assessment of treatment response and used very strict criteria to select 21 of 192 patients (11%) with cCR who were then carefully followed-up and compared to 20 patients with a pCR after resection. Only one patient in the non-operative group developed a LR after a mean follow-up of 25 months and underwent successful surgery. No statistical differences in long-term outcomes were seen between the groups regarding 2-year OS and DFS. Short-term functional outcomes, however, were better in the WW group, with improved bowel function scores, less incontinence, and patients avoiding permanent colostomy. Other non-randomized, prospective studies and retrospective case series have added to the growing evidence that the non-operative approach seems safe with excellent rectal preservation and pelvic tumour control [158]-[162]. Disease regrowth in patients previously identified as having had a cCR requires surgical salvage, which has also been shown not to compromise outcomes, as compared with patients who received immediate surgery after neoadjuvant CRT [159],[163].

Although some studies did not achieve such impressive results and many clinicians remained sceptical of the WW approach, dedicated centres have reported encouraging oncological and functional outcomes, proposing that patients should be subjected to rigorous follow-up more frequently than routine surveillance to ensure feasible and timely surgical salvage [157],[164][167]. Systematic reviews have been published [5],[154],[166] and they all show that WW is likely safe with the use of resection in patients with tumour regrowth. Having said this, one study [164] noted a worse survival and a higher incidence of distant tumour progression in patients in the WW group with local regrowth versus those without.

So, the use of non-operative management of RC has been increasing and should be carried out in centres with experienced multidisciplinary teams after a careful discussion with the patient about his or her risk tolerance and understanding.

There are still controversial issues related to WW, namely the prediction of cCR and the insufficient concordance with pCR, the role of scar/ residual mucosal abnormalities (RMA) biopsy or local excision, the best therapeutic regime for maximal response, the risk of distant metastasis if tumour regrowth, the timing of reassessment, the long-term outcomes and optimal surveillance options [169]. Patients should be informed that a small increased oncological risk of uncontrolled pelvic and metastatic disease exists, although the prognosis of patients with cCR is excellent even without surgery.

Total neoadjuvant therapy

Neoadjuvant radiotherapy with concurrent fluoropyrimidines followed by surgery and adjuvant ChT has been the standard treatment for the past years. The lack of evidence of ChT in the adjuvant setting in RC has led to the concept of total neoadjuvant therapy (TNT), in which the whole radiation and ChT are given preoperatively.

TNT has been developed to optimize delivery of systemic therapy aimed at micrometastases and to obtain higher pCR and ressecability rates through intensification of neoadjuvant therapy. The increased patient compliance to ChT prior to surgery and the prediction of response are also arguments for this tactic.

These strategies using neoadjuvant ChT before or following LCCRT or SCRT, the so-called induction and consolidation therapy, respectively, are being investigated in multiple trials. Possible benefits of using ChT first include the early prevention or eradication of micrometastases, less length of time patients need an ileostomy, improved tolerance and higher ChT completion rates.

Randomized controlled trials, retrospective analyses and systematic revisions have reported excellent outcomes with high pCR rates in patients undergoing TNT, either consolidation or induction [170] [142][171][172][173]. Having said this, the fact is that there are no studies comparing the different regimens and timings used on the various studies.

The German study CAO/ARO/AIO-12 [142] compared induction ChT followed by CRT with CRT plus consolidation ChT. A pCR was achieved in 17% versus 25% ($p<0.001$) in favour of the consolidation arm that had longer time until surgery and better CRT compliance. ChT tolerance was greater with the induction arm.

In 2020, the RAPIDO trial [143] compared a conventional arm of CRT followed by TME with an experimental one with SCRT followed by an 18-week period of consolidation ChT and surgery. Pathological complete response was 27.7% versus 13.8% (OR 2.40, CI 1.70-3.39, $p<0.001$) in the experimental and standard groups, respectively. Likewise, there was a 3-year distant progression of 19.8% versus 26.6% (HR 0.69, CI 0.53-0.89; $p=0.004$) and 3-year LR of 8.7% versus 6.0% (HR 14.5, CI 0.93-2.25, $p=0.10$), respectively. This study demonstrated a lower disease recurrence in patients treated with TNT with SCRT.

The PRODIGE-23 trial compared conventional CRT followed by TME and adjuvant ChT with intensification of neoadjuvant therapy through FOLFIRINOX followed by CRT, surgery and adjuvant ChT. There was a higher pCR rate in the experimental arm (11.7% versus 27.5%, $p<0.001$), higher DFS (HR = 0.69, CI 0.49-0.97, $p=0.034$), 3-year local progression-free survival (LPFS) (HR = 0.64, CI 0.44-0.93, $p<0.02$) with no difference in the 3-year OS (HR = 0.65, IC 0.40-1.05, $p=0.077$).

In both RAPIDO and PRODIGE trials treatment duration was higher in the experimental arm. No differences between experimental and standard groups were noted related to therapy tolerance, perioperative morbidity and mortality. The addition of ChT before surgery, either before or after CRT, increased the probability of cCR. Although both studies demonstrated significant impact on 3-year DFS, none presented advantages related to OS.

Last year, OPRA trial preliminary results were also published. This study randomized RC patients to induction or consolidation ChT and patients were restaged at 8-12 weeks post CRT. In this trial,

consolidation therapy had a higher cCR rate than induction. The difference might be related to the longer time interval between the end of radiotherapy and surgery, apparently a particularly important benefit factor in TNT.

The best option for TNT, either induction or consolidation (or both), has not been completely established. In sum, so far studies seem to show that a total neoadjuvant therapy in the form of consolidation ChT increases the probability of complete response.

Adjuvant chemotherapy after neoadjuvant chemoradiation and surgery

The neoadjuvant CRT approach theoretically commits patients to the entire three-component package of CRT, surgery and adjuvant therapy. After surgery alone for RC there seems to be a benefit for adjuvant 5-FU based ChT in terms of DFS and OS, which has not been shown following SCRT or LCCRT. In patients who received preoperative LCCRT and postoperative ChT there was no benefit in DFS in the ones with ypT0N0 or ypT3-4Nx disease. The greatest benefit happened in ypT1-2N0 disease [59].

The decision on postoperative ChT should be risk-balanced, taking into account both the predicted toxicity for a particular patient and the risk of relapse. It also remains unclear whether the initial clinical (yc) or pathological (yp) stage should be used to determine the risk/ benefit of adjuvant treatment. In general, downgrading in T or N stage has been recognized more as a prognostic factor of favourable outcome rather than predictive biomarker for adjuvant treatment. It is, therefore, reasonable to consider adjuvant ChT in RC patients after preoperative LCCRT/ SCRT with yp stage III and 'high-risk' yp stage II [59].

Candidate Colorectal Unit Protocols

In the quality of Surgical Coordinator of the Hospital Beatriz Ângelo Rectal Cancer Group, the PhD Candidate contributed to the conception of protocols and pathways for the RC patients, namely:

- Diagnostic and therapeutic approach in rectal adenocarcinoma
- Active surveillance in patients with RC with cCR post CRT

Recommendations according to Stage

Table 7 represents the current recommendations for treatment according to clinical stage [17].

Table 7 Choice of treatment within TNM risk category of RC cM0

Risk Group	TN substage	Possible therapeutic options
Very early	cT1 sm1 N0	- Local excision - If pT1 and no adverse features, LE is sufficient - If adverse histopathology (sm2/3, G3, EMVI, LVI) requires TME
Early (good)	cT1-cT2; cT3a/b if middle or high, N0 (or also cN1 if high), CRM clear, no EMVI	TME is standard. If unexpected poor prognostic signs on histopathology (CRM, extranodal/N2), consider postoperative CRT/ChT
Intermediate	cT3a/b very low, levators clear, MRF clear or cT3a/b in mid- or high rectum, cN1-2 (not extranodal), no EMVI	TME is a standard only if good quality mesorectal resection assured; if not, preoperative SCRT or CRT + TME
Bad	cT3c/d or very low localization levators threatened, CRM clear, cT3c/d mid-rectum, cN1–N2 (extranodal), EMVI, limited cT4aN0	SCRT or CRT + TME, depending on need for regression
Advanced (Ugly)	cT3 with any CRM involved, any cT4a/b, lateral node.	CRT + TME and more extended surgery if needed due to tumour overgrowth) or SCRT+ FOLFOX and delay to surgery

Recommendations for treatment according to European Society of Medical Oncology (ESMO)

CONCLUSION

Despite the great advances achieved in RC treatment many controversial issues still remain. Classic multimodal treatment with preoperative CRT has improved LR rates and, in some cases, allowed for sphincter preservation procedures. Subsequent TME is part of an optimal radical resection but now with the emphasis on specimen quality and achievement of a negative CRM.

However, recent studies start to question this traditional approach because of a number of issues. First, if cTNM staging was *per se* an indicator for CRT, studies have demonstrated that CRM status predicted by high-definition MR better depicts the indication for neoadjuvant therapies, as well as local and distant recurrence. Second, there is evidence that restaging after neoadjuvant CRT more accurately forecasts prognosis than initial clinical stage. Third, preoperative CRT indications have broadened and currently this option can also be considered in patients with T2N0 distally located tumours, in risk of sphincter loss. Likewise, strategies for achievement of better responses are being conducted, with intensification and TNT. Fourth, restaging and evaluation of response now dictates the pathway to follow with “Watch and Wait” possibly recommended for complete responders. Finally, because we cannot yet predict who will respond and who will not, there are concerns that, by submitting to CRT all patients clinically staged as T3N+, we might be overtreating some patients and delaying systemic treatment to 4-5 months after diagnosis, thereby increasing the risk to systemic dissemination.

Therefore, management of RC is clearly changing and it is imperative that patients are extensively discussed in a multidisciplinary team.

Chapter 3

Biological Determinants in the Treatment of Rectal Cancer

The role of miRNAs as Molecular Predictors of Response to Neoadjuvant Chemoradiotherapy

INTRODUCTION

Despite great progress in RC treatment options, CRT is still ministered in the majority of locally advanced cases and this is done to achieve downstaging and cCR, increase R0 resections, allow sphincter-sparing surgery and decrease local recurrence (LR) [59]. After neoadjuvant treatment, response can be quite variable. Almost 30% of patients develop cCR with no residual tumour identified, 40-60% achieve some degree of tumour downstaging, while 30% exhibit resistance to CRT having no benefit from this therapy [5]. In fact, non-responders are at increased risk of disease progression and unnecessary toxicities caused by CRT. Currently, we cannot predict response and the complications associated with this treatment should not be underestimated [5].

Recent data suggest that clinical complete responders can safely undergo a conservative approach without surgery [174]. By contrast, European Society of Medical Oncology (ESMO) guidelines recommend upfront surgery in T3a-bN1 tumours if there is no evidence of involvement of the CRM [59]. There is an urgent need to distinguish who will and who will not respond in order to individualize treatment, avoid CRT in predictable non-responders and elude mutilating resections in complete responders. Thus, pre-treatment prediction of response to CRT would be critical in deciding whether the patient should or not undergo this neoadjuvant therapy.

Currently, although molecular heterogeneity is a well-recognized feature of most tumours, CRC patients are still treated based solely on clinical stage. The inclusion of molecular markers in a treatment algorithm could potentially stratify patients, thus allow a better choice of candidates. However, no biomarkers have yet been validated for selection of patients for CRT. The “million-dollar” question is: can we find biomarkers of response to neoadjuvant CRT?

Biomarkers

Biomarker detection is a relatively non-invasive, convenient and economical method widely used in the clinical practice. According to the World Health Organization (WHO), “a biomarker is any substance, structure or process that can be measured in the body or its products and that influence or predict the outcome or disease” [175]. In other words, biomarkers are objective measures of deoxiribonucleic acid (DNA), ribonucleic acid (RNA), protein or any other molecule that describe a normal or pathogenic biologic process or pharmacological response to a therapeutic intervention [176].

The search for cancer biomarkers is performed to identify a specific disease during it’s early stage (diagnostic), assess the likely course of the disease (prognostic), evaluate treatment response (predictive) and potentially identify a target of therapy (therapeutic) [177].

In RC, despite the current interest in biomarker research, only conventional biomarkers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19.9 (Ca 19.9) and RAS gene mutations (K-RAS or N-RAS) are used the clinical setting [59]. Nevertheless, prognostic and predictive biomarkers could be extremely valuable to correctly select patients for treatment. The goal is to define a method for validation that assures that the biomarker can be measured reliably, precisely, and repeatedly at a low cost.

MicroRNAs in human cells

From the initial discovery of small RNAs in the model organism *Caenorhabditis elegans* in 1993 [178], it took some time for the acknowledgement of their relevance on pathological processes in humans. Considering the strong association between genetic alterations and neoplastic diseases, a new view of gene regulation was introduced in all fields of human biology and medicine, with a special focus on microRNAs (miRNA) and cancer.

In the first years, small interfering RNAs (siRNA) gained importance in experimental research, owing to the fact of being comfortable tools to silence the expression of certain proteins by translational repression without the need to engineer genetically modified cells. The enzymatic machinery of this "RNA interference" is, in fact, performed *in vivo* by small endogenous molecules, the so-called microRNAs (miRNA or miR).

miRNAs are short, 20-24 nucleotide (nt), non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of messenger RNA (mRNA) [179]. Highly conserved, miRNAs are found in plants, animals and humans and affect protein expression through inhibition of translation as well as degradation and destabilization of mRNA [180].

miRNAs are transcribed by RNA polymerase II as primary transcripts (pri-miRNAs) that can be either protein coding or non-coding. The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA). After nuclear export, another enzymatic process catalysed by DICER cytoplasmic ribonuclease further cleaves pre-miRNA to generate the mature miRNA. The mature miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA (Fig.1) [179][180][181].

miRNAs may exhibit an oncogenic or tumour suppressive effect depending on which genes or groups of genes they silence [182].

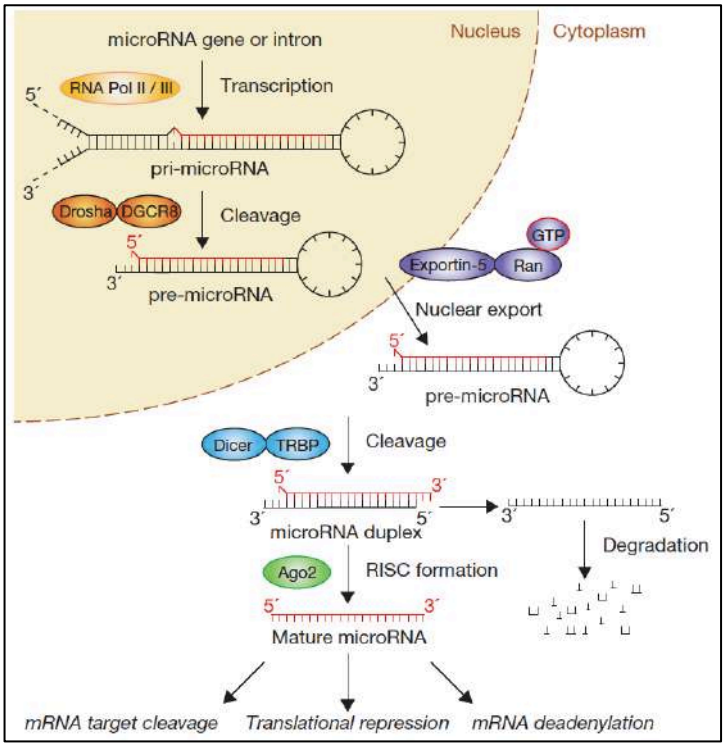

Figure 1 Diagram of biogenesis and function of miRNAs Initially, a primary transcript (pri-miRNA) is transcribed from the DNA by RNA polymerase. In a second step, a nuclear enzyme complex including the protein Drosha processes the primary transcript leading to a precursor-miRNA (pre-miRNA) that is exported from the nucleus to the cytoplasm. Here, the ribonuclease DICER cleaves the molecule to produce the "mature-miRNA" that is incorporated into the RISC. Finally, this complex mediates the inhibition of protein translation or the degradation of the target mRNA. Reproduced from *Winter et al. Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat Cell Biol 2009 Mar;11(3):228-34*

Up to 30% of the human genome is regulated by these molecules through influence in relevant cellular functions including tissue development, cell proliferation, cell differentiation, metabolism, stemness, stress response, angiogenesis, apoptosis, protein secretion and response to viral infection [182]-[187]. These processes interfere with major biological systems such as immunity and cancer [183],[184]. Although the precise biological role of many miRNAs is yet to be entirely elucidated, these new regulators of translation have already substantially changed our view of gene expression regulation.

MicroRNAs as biomarkers in colorectal cancer

The protagonism of miRNAs in colorectal carcinogenesis and tumour progression has been confirmed by numerous functional studies that have shown involvement in cell progression, invasion, angiogenesis and metastatic behaviour [182]-[187]. miRNAs can function as tumour suppressors or oncogenes, repressing the expression of important cancer-related genes. The role of these molecules in the

regulation of carcinogenic pathways hypothesized their use as biomarkers in cancer diagnostic and prediction of response to therapy [188].

miRNAs can be extracted from a wide variety of biological materials, including archival formalin fixed paraffin embedded (FFPE) tissues and body fluids collected in clinical settings (plasma, serum, saliva or stool) [189]. The stability of miRNAs in these various samples remains a strong motivation for their development as clinically useful biomarkers. In fact, miRNAs have a considerable higher potential as biomarkers when compared to nucleic acids such as DNA and mRNAs. miRNAs are well preserved in FFPE tissues even after archival times of up to 10 years [190]. On the contrary, long formalin fixation times tend to degrade DNA and mRNA decreasing the amounts extracted from FFPE tissues. The poor quality of mRNA and DNA obtained from FFPE samples makes its use limited for advanced molecular biology techniques complicating quantitative analyses. Another important obstacle for developing mRNA-based biomarkers is the presence of RNase in body fluids that tends to degrade mRNAs [189],[191].

Increasing evidence indicates that aberrant expression of miRNAs is present in different types of cancers, including colorectal, lung, breast, pancreatic, cervical, ovarian and prostate cancer [192]-[196]. In fact, expression patterns can be unique to specific cancers offering capability of diagnosis in early stages. Regarding prediction of treatment, it has been demonstrated that many cases of ChT failure are induced by aberrant expression of miRNAs in various cancers.

In CRC, miRNAs have a role in distinguishing normal tissue from adenoma and carcinoma [197],[198], as subtypes of tumours, namely microsatellite unstable high (MSI-H) cases [199],[200]. MSI-H colorectal cancers make up to 15% of all sporadic CRC and harbour defects in the mismatch repair system. In the inherited Lynch syndrome, mismatch repair proteins carry germline mutations. miRNA profiles have been shown to separate MSI-H from microsatellite stable (MSS) cancers with a specificity of around 80% and a sensitivity of around 90% [199],[200]. Although similar in conventional histology, these cancer forms contrast in prognosis and response to ChT.

miRNA associated with CRC have been identified in tumour tissue but the need for a non-invasive tool prompted their investigation in serum and plasma as circulating markers. Deregulated miRNAs were, indeed, found in plasma and serum at detectable levels [201],[202]. It seems that CRC-derived miRNAs are released into the blood stream so plasma miRNA expression can reflect the signature of the tumour tissue. miRNA released into systemic circulation, freely or in exosomal shells, is less vulnerable to RNase-mediated degradation, remaining remarkably stable, reproducible and not degraded for a long half-life, withstanding several repeated freeze-thaw cycles [203].

The idea of a correlation between circulating and tissue miRNA supports the hypothesis that plasmatic miRNAs can serve as biomarkers of disease or disease response to therapy. Direct identification of circulating miRNAs as liquid biopsies can provide information for diagnosis, prognosis and predictive responses to treatment in CRC without the need for tumour-tissue biopsies [203]. This unique feature is also a central reason for the recent interest of miRNA biomarker studies in the field of cancer research.

MicroRNA and chemotherapy resistance

It has been shown that miRNAs play vital roles in ChT resistance in multiple cancer cell lines, its expression being significantly altered following treatment with 5-FU [204]. This influence in response happens through various mechanisms [195],[196],[204], including: 1) targeting enzymes involved in 5-FU metabolism reducing sensitivity to the drug; 2) apoptosis signalling pathways and autophagy; 3) epithelial–mesenchymal transition (EMT) program and 4) cell stemness.

5-FU is a cell cycle–specific agent that generates several metabolites affecting the S phase of proliferation contributing to disruption of DNA and RNA synthesis. The enzymes responsible for 5-FU metabolism include thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP). DPD is the initial and rate-limiting enzyme in the catabolism of 5-FU, functioning to convert 5-FU to dihydrofluorouracil (DHFU) [207]. The molecular mechanisms of 5-FU resistance seem to be associated with high expression of these enzymes, influenced by miRNA [208],[209].

Apoptosis resistance is a major hallmark of cancer and many miRNAs have been shown to be involved in regulating apoptotic-signalling pathways, affecting 5-FU sensitivity [210], [211].

miRNAs also interfere with the epithelial–mesenchymal transition (EMT) program that converts epithelial cells into mesenchymal cells which acquire stem cell–like features. Growing evidence supports that cancer cells enter the EMT process and gain cancer stem cells (CSC) pluripotency [212]. Once cancer cells acquire this capacity, resistance to medical therapies increase and metastasis can occur.

Cancer stem cells (CSC), thought to give rise to tumours and to be involved in the development and progression of cancer, have also been shown to influence 5-FU resistance. CSCs are a minority population of cells within a tumour, characterized by self-renewal and high tumorigenic capacity. These cells increase following administration of ChT and radiotherapy, supporting the concept of being resistant to conventional therapies and offering a potential explanation for treatment failure [208]. In epithelial tumours such as RC, CSC phenotype and EMT program cooperate to impact tumour progression, metastasis and therapeutic resistance [205],[209],[213]. In this setting, CSCs have gained interest as targets to overcome non-response to traditional cancer therapies such as ChT and radiation.

MicroRNAs and Response to Radiotherapy

The use of radiotherapy in cancer treatment is based on the fact that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro* and inhibit tumour growth *in vivo* [214]. However, tumour response to radiotherapy sometimes differs even among neoplasia with the same histological background.

The majority of the identified genes related to radiosensitivity in human cancer have been associated with apoptosis, DNA-repair, growth factors, signal transduction, cell cycle, cell adhesion, invasion, angiogenesis and hypoxia. An association between radioresistance and the expression of several genes was observed, namely p53, RAS, raf-1, bcl-2 and survivin [215]-[217]. Likewise, CSCs have emerged as contributing to radioresistance through the preferential activation of the DNA damage checkpoint response and the increase in DNA repair capacity [218].

Although there is a partial understanding of the molecular mechanisms responsible for cellular radiosensitivity, the entire process remains to be elucidated. In fact, the identification of the determinants of the tumour response to radiation has long been a goal of radiation oncologists and biologists. In RC, the lack of knowledge regarding biomarkers of response has limited the selection of patients for neoadjuvant CRT. The discovery of miRNAs has substantially changed the view on gene regulation but, as pathways become more complex, new questions arise. miRNAs interfere with genes involved in the radioresistance but, in contrast to colon cancer, in RC very limited data is available on their impact in tumour response, with studies approaching only 5-FU based therapies and not capecitabine. Also, the potential of circulating miRNAs as biomarkers in this setting is still scarcely investigated.

In summary, if found to be associated with response to CRT, miRNAs could potentially be used as biomarkers to predict it and to guide therapeutic decision-making in RC patients. This is the fundamental question on the basis of this Chapter: can miRNAs predict response to CRT in RC patients?

Specific microRNAs in colorectal cancer

Due to their characteristics, miRNAs are involved in the regulation of carcinogenic pathways, including in CRC [213],[214],[219]. Aberrant levels of miRNAs have been associated with the initiation, progression, and drug-resistance of CRC. Several studies have demonstrated the tumour suppressive or oncogenic functions of miRNAs and their applications in the clinical setting as biomarkers or therapeutic targets for CRC. So, specific miRNAs have been demonstrated to be potential biomarkers for CRC (Fig.2) and miR-16, miR-21, miR-135 b, miR-145 and miR-335 often appear as the most studied miRNAs [178]

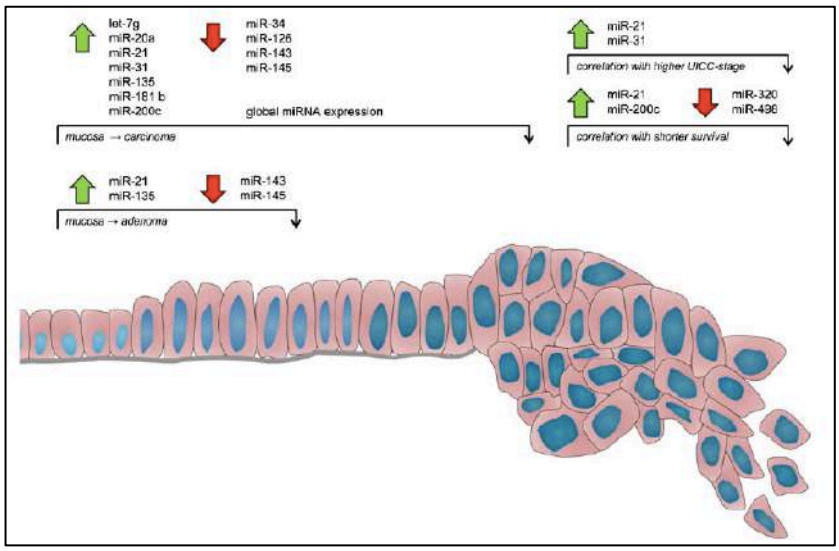

Figure 2 Changes of miRNA expression in colorectal adenomas, carcinomas and their correlation with stage or survival. Several specific miRNAs are up- or downregulated in adenomas and carcinomas compared to normal colorectal mucosa, based on expression profiling experiments. Additionally, expression levels of some miRNA show a correlation with different stages and survival in CRC. Only a selection of miRNAs is shown. Reproduced from *Faber et al. The impact of microRNAs on colorectal cancer, Virchow's Arch (2009) 454:359–367*

MicroRNA-16

MicroRNA-16 (miR-16) has been found to function as a tumour suppressor in a variety of human cancers and its dowregulation can promote CRC cancer [219].

The development and progression of CRC is a complicated process that involves the deregulation of several genes essential for cellular processes. Members of the GTPases of the RAS family (KRAS, HRAS and NRAS) are well known for their ability to cause neoplasia [197]. Among the RAS family, KRAS is one of the most prominent oncogenes due to its ability to transform human cells into malignant ones, particularly when harbouring an activating mutation in codon 12 or 1337. KRAS mutations occur in 30–60% of CRC and are often associated with tumour resistance to ChT and targeted therapies [220].

KRAS is a direct target of miR-16 that acts as a tumour suppressor through the inhibition of KRAS translation. There is evidence of a regulatory network between miR-16 and KRAS that controls cell proliferation, invasion and apoptosis in CRC cells. Silencing KRAS expression inhibit these processes while overexpression has the opposite effects, validating the role of KRAS as a crucial oncogene in CRC tumorigenesis [221] .

Equally, miR-16 directly inhibits vascular endothelial growth factor A (VEGFA). VEGFA activates vascular endothelial growth factor receptor 1 (VEGFR1) and together they activate ERK1/2, JNK MAPK and the phosphoinositide 3-kinase/AKT pathways that play critical roles in cancer proliferation, metastasis, survival, and angiogenesis [221],[223],[224]. Also, it has been reported that miR-16 can inhibit proliferation and induce apoptosis of CRC cells by regulating the p53/ survivin signalling pathway through the intrinsic apoptosis pathway (Fig.3) [221]. Also, as said before, Ras, p53, survivin are involved in radiotherapy resistance.

Clinically, low miR-16 expression in CRC tissue is significantly associated with tumour undifferentiation, higher incidence of LN metastasis, advanced TNM stage and higher incidence of tumour recurrence. Equally, miR-16 expression is an independent prognostic factor with low expression predicting poorer prognosis and survival (HR 1.67; 95 % CI 1.22–2.54; p = 0.018) [224].

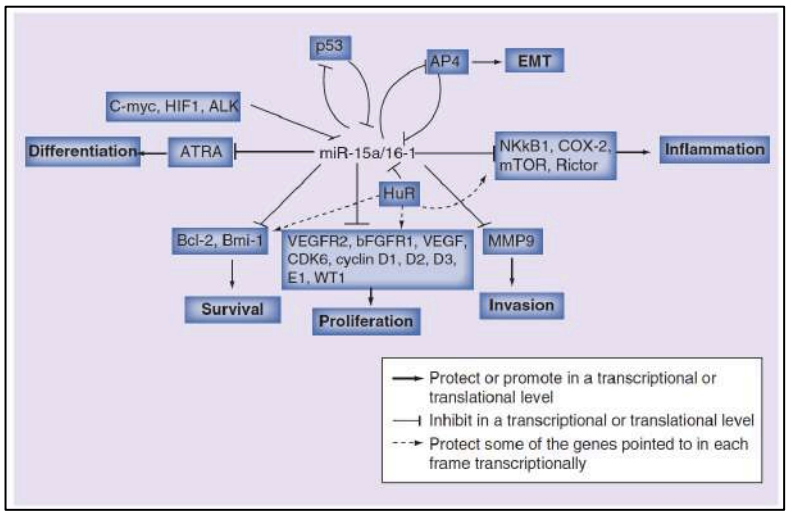

Figure 3 Diagram of biogenesis and function of miRNA-16. Reproduced from *Huang et al, MicroRNA 15a/16: as tumour suppressor and more. Future Oncol. 2015;11(16):2351-63.*

MicroRNA-21

microRNA-21 (miR-21) is one of the most well-established oncogenic miRNAs, up-regulated in CRC [186]. miR-21 plays a key role in several biological processes needed for tumorigenesis, including resistance to apoptosis, invasion, cell proliferation, evasion to growth suppressors, replicative immortality and inflammation [186]. It contributes to intracellular signalling cascades, positive regulation of angiogenesis, interphase of mitotic cell cycle and negative regulation of cell differentiation. In addition, this miRNA has been shown to affect genetic instability, metastization and resistance to ChT in several solid tumours including CRC [180], [194],[218]-[223],[177],[191].

miR-21 oncogenic function is exerted mainly through the suppression of various genes that participate directly or indirectly in the extrinsic or intrinsic apoptosis pathways, namely programmed cell death (PDCD4), phosphatase and tensin homolog (PTEN) or RAS/ epidermal growth factor (EGF). miR-21 is also a negative regulator of p53 signalling and promotes nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), implicated in controlling DNA transcription [186][221],[224]-[227]

Overexpression of miR-21 enhances cell proliferation trough targeting PTEN and phosphatidylinositol-3-kinase-AK (PI3-K-AKT), while decreasing apoptosis via targeting BTG2, FasL and FBXO11. The PI3-K-AKT pathway is a central oncogenic mechanism involving the regulatory subunit p85-alpha, frequently mutated in CRC [235]. The tumour suppressor gene PTEN is an important inhibitor in this pathway [236]. Apart from interference with the PI-3-K-AKT pathway, miR-21 has been shown to act on PDCD4 gene, a tumour suppressor gene that is an independent prognostic factor in colorectal cancer with an inverse correlation between levels of miR-21 and PDCD4 protein (Fig.4) [232],[237]. Silencing of miR-21 by anti-miR-21 resulted in increased levels of PDCD4 in CRC cell lines. In addition, CRC tumour tissue shows higher miR-21 expression and decreased amounts of PDCD4 protein than the normal mucosa. These results argue for an important function of miR-21 in the pathogenesis of CRC, as it also shows a good correlation with prognosis [178],[238]. Additionally, by targeting PTEN and PDCD4, miR-21 has been shown to regulate mitogen-activated protein kinases (MAPK) and WnT/β-catenin pathways, which play a central role in early colorectal tumour development [231].

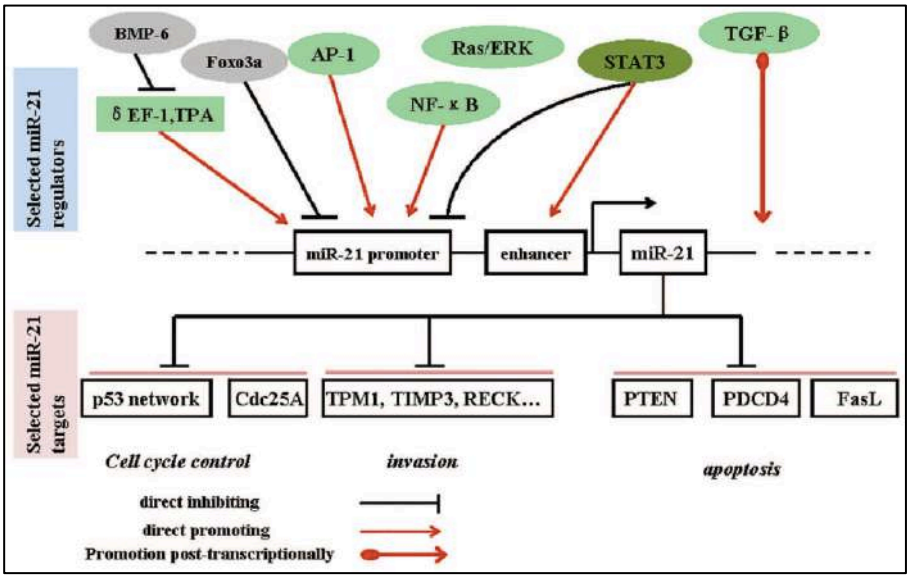

Figure 4 The upstream regulation of miR-21 and the validated targets of miR-21 in human cancer.
Reproduced from *Pan et al, MicroRNA-21: A novel therapeutic target in human cancer. Cancer Biology & Therapy 10:12, 1224-1232; December 15, 2010*

Studies report miR-21 as a screening, diagnostic and prognostic biomarker in CRC. This molecule is frequently overexpressed in CRC in comparison with adenoma tissues and in these in relation to normal mucosa [187],[229],[230]. Likewise, miR-21 is significantly up-regulated in both plasma and matched tissue of CRC samples compared to healthy controls [144]. Overall, the expression of this miRNA has been reported to be associated with clinical stage, up-regulation being related to advanced stage, positive LN, venous invasion, metastatic behaviour and poor survival [144],[188],[232],[237],[230].

Finally, miR-21 has emerged as a potential biomarker for chemoradiotherapy sensitivity in CRC *In vitro* [223],[231]. Overexpression of miR-21 in colon cancer cell lines has been described to induce resistance to 5-FU and inhibit their sensitivity to irradiation. Moreover, the upregulation of miR-21 has been shown to reduce the efficacy of 5-FU in xenograft mice by reducing the expression of enzymes involved in 5-FU metabolism. This could be indicative of a possible mechanism of resistance to 5-FU-based CRT in RC. Also, targeting miR-21 reduces the number of CRC CSCs during 5-FU treatment [226],[234].

MicroRNA-135b

Other miRNA known to act as an oncogene is microRNA 135b (miR-135b), involved in cancer progression, metastasis and invasion. This miRNA modulates cell proliferation, apoptosis and chemoresistance through regulating key tumour suppressor genes such as Adenomatous Polyposis Coli (APC) [242]. miR-135b suppresses APC expression decreasing the translation of this gene transcript and inducing β-catenin/Wnt pathway. More than 60% of all colorectal adenomas and carcinomas carry a mutation in the APC gene and high miR-135b levels correlate with low APC levels (Fig. 5) [243],[244].

An important target of miR-135b in CRC is Forkhead box O 1 (FOXO1). FOXO1 is a transcription factor that participates in a variety of biological processes, including DNA repair, cell cycle transition, apoptosis and oxidative stress response. At the downstream, FOXO1 promotes transcriptional activities of Bim and Noxa, pro-apoptotic proteins. Therefore, FOXO1 is a key regulator of apoptosis acting as a tumour suppressor. Overexpression of miR-135b induces downregulation of FOXO1, which was found to promote cell proliferation, tumorigenesis and be responsible for low sensitivity of CRC cells to oxaliplatin treatment [246],[247] .

In CRC, the expression of miR-135b is significantly upregulated both in tissue and plasma samples of patients with colorectal adenomas and carcinomas in comparison with healthy controls, correlating with tumour stage and poor clinical outcome [244],[247].

In this context, some studies suggest that miR-135b acts as a contributor of chemoresistance with anti-apoptotic effects. It was shown that *in vitro* overexpression of miR-135b attenuates the apoptosis rate of colon cancer cell lines in response to 5-FU treatment, while inducing its proliferation [239]. Moreover, studies with anti-miR-135b demonstrated to enhance the oxaliplatin-induced cytotoxicity in colon cancer cell cell lines and in xenograft mice, thus indicating that decreasing miR-135b levels may lead to an increase in sensitivity to oxaliplatin treatment [241].

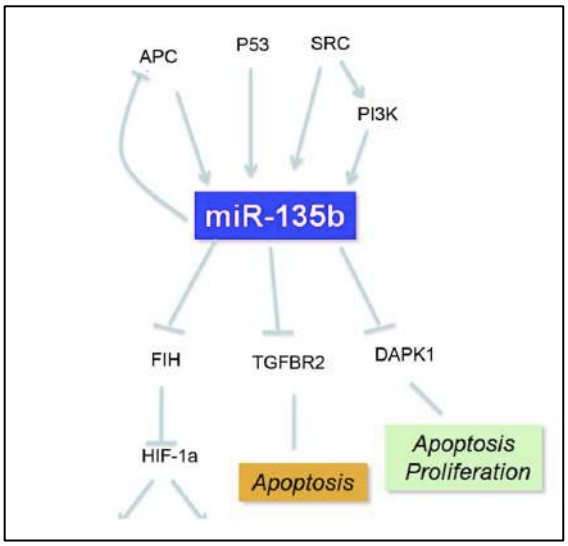

Figure 5 Overview miR-135 involvement in key signalling pathways in colorectal cancer. Reproduced from *Valeri et al, MicroRNA-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. Cell 2014 Apr 14;25(4):469-83*

MicroRNA-335

The action of some miRNAs in CRC carcinogenesis has not yet been fully elucidated and their role is still controversial, which is the case of microRNA-335 (miR-335).

miR-335 is acknowledged to act as tumour suppressor by regulating expression of ZEB2, an epithelial-mesenchymal transition (EMT)-related gene that functions inducing EMT and facilitating development of metastasis of cancer cells. miR-335 directly regulates ZEB2 expression by inducing mRNA degradation and translational suppression. Furthermore, the most important effect exerted by miR-335 on cell invasion and migration is partially reversed after transfection with a ZEB2 expression vector [204],[214].

Overexpression of miR-335 inhibits CRC migration, invasion and metastization. In fact, expression of miR-335 correlates inversely with the clinical stage and downregulation is associated with the aggressive phenotypes of CRC, LN positive cases, poor and shorter OS [178].

MicroRNA-145

MicroRNA-145 (miR-145) was the first miRNA reported, with reduced levels being associated with CRC development [249], involved in the regulation of various cellular processes such as the cell cycle, proliferation, apoptosis, angiogenesis and invasion [249].

miR-145 is a tumour suppressor that targets the insulin receptor substrate-1 (IRS-1) and type 1 insulin-like growth factor receptor (IGF-IR) [250]. IGF-IR is an EMT mediator that regulates growth and differentiation of normal and abnormal cells. This receptor has a confirmed role in cancer, dramatically stimulating the growth of cancer cells, so its down-regulation (by antisense or antibodies) causes inhibition of tumour growth *in vitro* and *in vivo*, with cells accumulating in the G2 phase of cell cycle [250]. IRS-1, especially when activated by the IGF-IR, sends a strong mitogenic, anti-apoptotic and anti-differentiation signal directly involved in tumorigenesis of CRC. IRS-1 increases transcription of rDNA, c-myc, cyclin D1, and cf/Lef promoters, stimulators of cell cycle progression genes [250].

miR-145 also directly downregulates other EMT mediators such as fascin-1 and paxillin, suppressing cell proliferation and metastization, as well as stem cell transcription factors including c-Myc, KLF4, Oct4, Nano and the Snail Family Transcriptional Repressor 1 (SNAI1). miR-145 expression is, in fact, inversely correlated with EMT [251].

The Snail Family Transcriptional Repressor 1 (SNAI1) is a transcriptional factor and a cancer stem cell (CSC) biomarker that has a critical role in driving the EMT program. SNAI1 level is consistently elevated in RC tissue and its overexpression sustains stemness, conferring a radiation resistant phenotype and decreased oxaliplatin sensitivity. Likewise, highly expressing SNAI1 cells are able to induce the expression of Nanog which also confers a radiation resistance. Overall, CSCs have increased expression of SNAI1, SNAI2, Nanog, c-Myc and IGF-1R, mediators for self-renew ability, but a significant decrease in miR-145 levels. On one hand, miR-145 inhibits both SNAI1-mediated stemness and SNAI1 driven expression of other critical CSC transcription factors. On the other, SNAI1 represses miR-145 activity, so cells with high SNAI1 have low miR-145 expression [178],[182],[253].

This relation between SNAI1 and miR-145 regulates the expression of CSC transcriptional factors in order to modulate the response to radiation. miR-145 can sensitize SNAI1 overexpressing cells to radiation therapy and to oxaliplatin. In fact, miR145 is overexpressed in post-CRT tissues in comparison with pre-CRT, with significant correlation with tumour regression [250]. So, miR-145 delivery represents a promising strategy to improve neoadjuvant therapy and overcome radiation resistance in RC. In sum, miR-145 represents a key molecular regulator of both the CSC phenotype and SNAI1-mediated radiation resistance (Fig 6).

Finally, miR-145 also functions as a suppressor of cell proliferation and tumour metastasis targeting oncogenes KRAS, MUC1, MAPK, EGF-R, HOXA9, STAT1, TGFBRE, APC, STA, YES1, FLI1, c-Myc, SOX2 [181],[223],[254]. miR-145 decreases HIF-1a expression, a major transcriptional regulator of VEGF in response to hypoxia, and decreases VEGF expression, leading to the inhibition of tumour growth and angiogenesis. Concurrently, miR-145 expression is increased by the tumour suppressor p53.

Clinically, miR-145 expression levels in CRC are associated with tumour stage, depth of invasion (pT), lymph node status (pN), development of distant metastases, grade of tumour differentiation, maximal tumour diameter, anatomical site and seric CEA levels. This miRNA is significantly reduced in plasma and tissue samples at the adenomatous and cancer stages of colorectal neoplasm in comparison with normal mucosa, supporting a role in early stages of carcinogenesis [243],[254]. Finally, it also seems to have an effect in metastatic cancer and reduced expression of miR-145 is associated with a worse prognosis [227], [255].

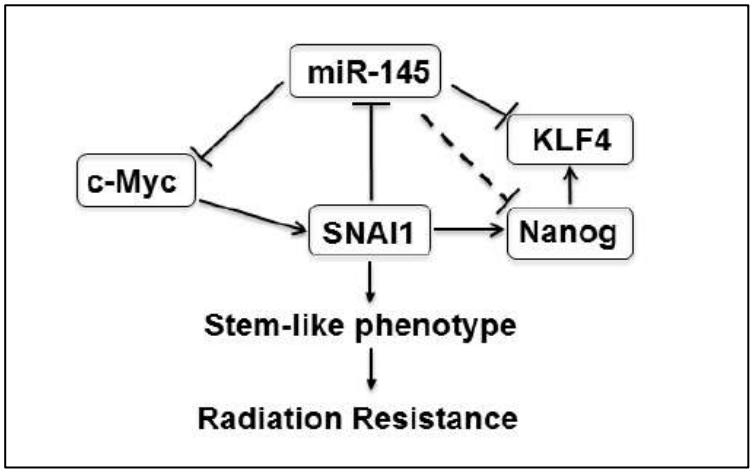

Figure 6 Overview of miR-145 involvement in key signalling pathways in colorectal cancer, their inductors and their targets. Reproduced from *Faber et al. The impact of microRNAs on colorectal cancer, Virchow's Arch (2009) 454:359–36*

In sum, deregulation of miRNA expression and correlation with histopathology indicate a biological role in RC development. As shown, miRNAs have been associated with response to therapy. However, single miRNAs alone are not considered ideal biomarkers since these molecules are not usually specific for one type of cancer. The combination of different miRNAs has been investigated and studies have tried to find associations between single or a panel of miRNAs and clinical features, supporting the idea that miRNA expression combined with parameters such as disease stage, tumour characteristics or even other biomarkers could be a better method to predict response to treatment (Table 1).

Table 1 Deregulated miRNAs in CRC and their principal targets

MiRNA	***Deregulation***	***Target gene***	***Effect***
miR-16	Downregulated	VEGFR, COX-2, KRAS	Suppress cell proliferation, growth
miR-17	Upregulated	Par4	Promote cell proliferation, reduce apoptosis
miR-18a	Downregulated	CDC42	Inhibit CRC cell growth, death
miR-19b-1	Downregulated	ACSL/SCD	Inhibit invasion
miR-21	Upregulated	PDCD4, PTEN, PI3KAKT	Inhibit apoptosis, promote cell survival
miR-30a	Downregulated	Metadherin	Inhibit cell migration, invasion
miR-106a	Upregulated	PTEN	Promote cell proliferation, reduce apoptosis
miR-135b	Upregulated	APC, FOXO1	Promote cell growth, migration, invasion
miR-145	Downregulated	IRS-1, IGF-IR, SNAI1	Suppress cell proliferation, growth
miR-155	Downregulated	CTHRC1	Suppress cell proliferation, promote, apoptosis
miR-186	Downregulated	ZEB1	Inhibit cell proliferation, metastasis, EMT
miR-216a	Downregulated	COX-2/ALOXS	Suppress cells proliferation
miR-221	Upregulated	TP53INP1	Promote cell proliferation, reduce apoptosis
miR-335	Downregulated	ZEB2	Inhibit cell proliferation invasion
miR-383	Downregulated	PAX6	Inhibit cell proliferation, invasion
miR-494	Upregulated	APC	Promote cell growth
miR-511	Downregulated	HDFG	Inhibit cell proliferation, invasion
miR-598	Upregulated	INPPSE	Promote cell proliferation, cycle progression
miR-744	Downregulated	Notch1	Inhibit cell proliferation, invasion
miR-1271	Downregulated	Capn4	Inhibit cell proliferation, invasion
miR-1273g	Upregulated	CNR1	Promote cell proliferation, migration, invasion

Original Paper 2

Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma

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ABSTRACT

***Background**: Patients with locally advanced rectal adenocarcinoma are treated with neoadjuvant chemoradiotherapy. However, biomarkers for patient selection are lacking and the association between miRNA expression and treatment response and oncological outcomes is unclear.*

***Objectives**: To investigate the association between miRNA expression and response to neoadjuvant chemoradiotherapy and oncological outcomes.*

***Methods**: This retrospective study analysed miRNA expression (miR-16, miR-21, miR-135b, miR-145 and miR-335) in pre- and post-chemoradiation rectal adenocarcinoma tissue and non-neoplastic mucosa in 91 patients treated with neoadjuvant chemoradiotherapy (50.4Gy) and proctectomy. Two groups were defined: a pathological complete responders group (Tumour regression grade - TRG 0) and a pathological incomplete responders group (TRG 1, 2 and 3).*

***Results**: miR-21 and miR-135b were up regulated in tumour tissue of incomplete responders comparing with non-neoplastic tissue ($p = 0.008$ and $p < 0.0001$, respectively). Multivariate analysis showed significant association between miR-21 in pre-chemoradiotherapy tumour tissue and response, with a 3.67 odds ratio (OR) of incomplete-response in patients with higher miR-21 levels ($p = 0.04$). Patients treated with 5-fluorouracil presented reduced odds of incomplete response compared with those treated with capecitabine (OR = 0.19; 95% confidence interval 0.03 - 1.12, $p = 0.05$). Significant differences were seen in overall survival in relation to clinical TNM stage ($p = 0.0004$), cT ($p = 0.0001$), presence of distant disease ($p = 0.002$), mesorectal tumour deposits ($p = 0.003$) and tumour regression grade ($p = 0.04$), with a borderline significance for threatened mesorectal fascia ($p = 0.05$). A close to significant association was found between risk of death and higher miR-21 expression (HR 2.68; 95% CI 0.86 - 8.36, $p = 0.09$).*

***Conclusion**: miR-21 may predict response to chemoradiotherapy in rectal cancer.*

Keywords: rectal cancer, chemoradiotherapy response, tumour regression grade, miR-21, biomarkers

RATIONAL AND AIMS

Rational: The known influence of microRNAs in the mechanisms of CRC carcinogenesis and resistance to ChT raises interrogations related to their role in response to CRT and radiotherapy resistance. The stability of miRNAs and easy extraction from tissue samples, both fresh and formalin fixed paraffin embedded, is a strong motivation for their development as clinically useful biomarkers. This study hypothesised that the expression of specific miRNAs in RC tissue could be associated with response to CRT, being able to predict it and differentiate responders from non-responders.

Aims: This study aimed to investigate miRNAs as predictors of pathological response to CRT in RC. Based on literature review including our Group's previously published data [256], five miRNAs were chosen by virtue of having been demonstrated to be potential biomarkers for CRC. Thus, tissue miR-16, miR-21, miR-135b, miR-145 and miR-335 expression was determined and correlated with both pathological response and oncological outcomes.

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MATERIALS AND METHODS

Patients and tissue samples

This was a retrospective study of prospectively analysed data and samples. Patients with rectal adenocarcinoma (stage II-IV) diagnosed between March 2013 and September 2017 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal) treated with LCCRT and proctectomy were eligible.

Patients had a preoperative staging with pelvic MR, thoraco-abdominal CT and ERUS when pelvic MR was not clinically possible. Histopathological features were confirmed by pathological analysis and patients were staged according to TNM staging system (8th edition, 2017).

Patients with other histological types of rectal malignancy, not submitted to CRT or surgical resection, pregnant or under the age of 18 were excluded. Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institution's Human Research Committee and Ethical Committee on 13th March 2017. The study was registered in the Portuguese Data Protection Agency.

Neoadjuvant treatment

All patients underwent neoadjuvant LCCRT consisting of a 2Gy daily fraction of pelvic irradiation, 5 times a week, in a total of 50.4Gy. Radiation was delivered with capecitabine (825 mg/m2/twice daily, continuously during RT) or 5-FU (225 mg/m2 continuous infusion during RT). All patients except for 1 received more than 80% of the planned radiotherapy with a curative intent. Surgery was performed 10 to 12 weeks after CRT.

Assessment of pathological response

Tissue was retrieved from formalin fixed paraffin embedded (FFPE) samples. Histological confirmation of the biopsy samples was done by pathologist review and neoplastic and adjacent normal rectal tissue were differentiated based on hematoxylin and eosin (H&E) stain. A fixed amount of tissue (80 µm) across the samples was extracted for RNA isolation.

Pre-CRT RC biopsies (colonoscopy) were obtained from complete and incomplete responders as well as post-CRT tumour tissues (protectomy specimen) from incomplete responders. To allow a direct comparison of RC to matched non-neoplastic rectal mucosa, we collected adjacent (> 1 cm distant) non-tumour tissue both in biopsies and protectomy specimens (Fig. 7)

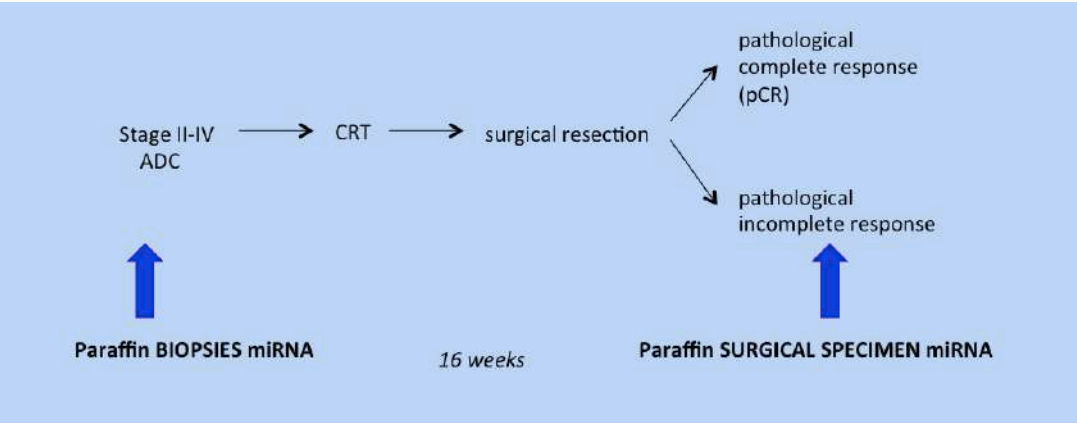

Figure 7 Timings of tissue sampling

Pathology specimens were graded by TRG according to the College of American Pathologists (CAP) guidelines. TRG was assessed by 2 pathologists blinded to patients clinical data and was categorized as TRG 0 (no viable tumour cells or complete response), TRG 1 (single cells or little groups of cancer cells), TRG 2 (residual cancer outgrown by fibrosis) and TRG 3 (minimal or no tumour kill with extensive residual cancer). Two groups of patients were defined, including a pathological complete responders group (TRG 0) and a pathological incomplete responders group (TRG 1, 2 and 3).

Table 2 Pathological TRG according to CAP

CAP TRG Score	
0	No viable residual tumour (complete response: pCR)
1	Marked response (minimal residual cancer with single cells or small groups of cancer cells)
2	Moderate response (residual cancer outgrown by fibrosis)
3	Poor or no response (extensive residual cancer)

RNA isolation

For total RNA isolation, pre- and post-CRT FFPE non-tumour and tumour rectal tissue samples were first deparaffinized with xylene (VWR International, Radnor, PA, USA) in two washing steps at 50°C. The samples were then fully homogenized into fine particles in 100% ethanol using a motor-driven grinder and centrifuged at maximum speed for 5 min. The collected pellet was rehydrated with 95% ethanol for 10 min following a new centrifugation step at maximum speed for 5 min. Then, samples were lysed with 500 µg/mL proteinase K in 100 µL of protease digestion buffer (20 mM Tris-HCl pH 8.0, 1 mM CaCl2 0.5 % SDS) at 55°C. Total RNA was isolated using RibozolTM reagent (VWR International, Radnor, PA, USA) according to the manufacturer's instructions and eluted into 20 µL RNase-free water. For a better evaluation of miRNAs quantity in total RNA, the miRNA concentration was determined using QubitTM miRNA Assay kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA).

Expression analysis by real-time PCR (RT-PCR)

cDNA synthesis was performed using TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For a uniform quantification of the quantity of miRNA to be used in cDNA, 2 µL of total RNA (corresponding to 2 ng of RNA) were extended by a 3' poly-A tailing reaction and a 5adaptor ligation to the mature miRNAs. miRNAs were reverse-transcribed into cDNA by reverse transcription using Universal RT primers. In order to improve detection of low-expressing miRNA targets, a pre-amplification of the cDNA was performed using the Universal miR-Amp Primers and miR-Amp Master Mix to uniformly increase the amount of cDNA for each target, maintaining the relative differential expression levels. cDNA samples were stored at -20°C.

Real-time polymerase chain reaction (PCR) was performed on a QuantstudioTM 7 Flex real-time PCR instrument (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) with TaqManTM Advanced microRNA Assays (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) to assess the expression profile of hsa-miR-16-5p (Assay ID 477860_mir), hsa-miR-135b-5p (Assay ID 478582_mir

hsa-miR-145-5p (Assay ID 477916_mir), hsa-miR-335-5p (Assay ID 478324_mir) and hsa-miR-21-5p (Assay ID 477975_mir). All reactions were performed in duplicate.

Due to the fact that a consensual endogenous control for miR expression in rectal tissue has still not been determined, initial preliminary analyses were performed to test several miRNAs as controls. Normalization was then performed with hsa-miR-484 (Assay ID 478308_mir), identified as the most stably expressed miRNA with the lowest expression variability between samples in these patient data set when compared with mir-1228-5p, miR-345-5p, miR-103a-3p, small nuclear (snRNA) U6 and RNU6B, considered controls for CRC tissues. Expression levels were calculated by the threshold cycle (2-ΔΔCt method) where ΔΔCt = (CT target miR – Ct control) sample – (CT target miR – CT control) median, when amplification values were detected in the real-time PCR. Due to lack of amplification values detected by the real-time PCR in all patient tissues, a variable number of samples were included in each miRNA expression profile.

Statistical analysis

The estimated sample size was 86 patients (43 patients per group of low and high miR expression). Sample size was calculated with an estimated proportion of patients TRG 0 with high and low miR-21 expression of 0.067 and 0.35, respectively. Type I and type II errors were set at α=0.05 and β=0.2, respectively. miRNA expression was analysed using the Graph Pad Prism software package, version 7.0 (GraphPad software Inc., San Diego, CA, USA). Normal distribution was determined using the D'Agostino & Pearson omnibus test. Data was analysed according to normality of values distribution using the one-way analysis of variance (ANOVA) followed by Kruskal-Wallis non-parametric Dunn's multiple comparison test or ANOVA Tukey's multiple comparisons test according to Gaussian distribution.

Receiver operating characteristic curve (ROC) analysis was then conducted, establishing the optimal cut-offs for each miRNA before CRT in normal and tumour tissue, determined as the point closest to the top left part of the plot with perfect sensibility and sensitivity. All miRNAs were dichotomized according to these cut-offs. Further analysis was also performed to explore the best discriminative cut-off point for miR-21 by comparing the cut-off determined in this study (1.18) with a previously reported miR-21 cut-off (2.8) [257]. Both cut-offs presented a similar Area Under the Curve (AUC), with our cut-off having a AUC value of 0.65 (95 % CI = 0.518 - 0.790), a higher specificity (66 *versus* 60%), a lower sensitivity (64 *versus* 87%), a similar positive predictive value (PPV) (92 *versus* 90%) and lower negative predictive value (NPV) (29 *versus* 43%) (Figure 8). Although both dichotomizations presented similar performance, we chose the cut-off determined in this study that yielded a better-distributed categorization of miR-21. There was professional statistical review performed in this manuscript.

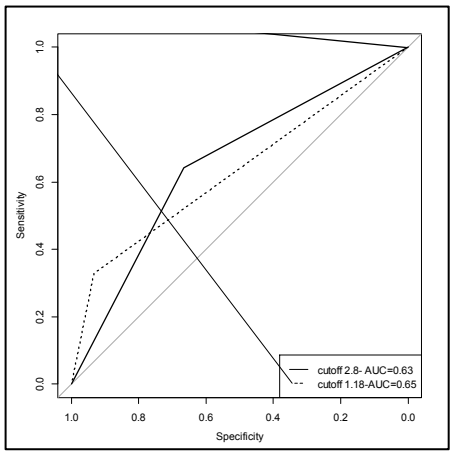

Figure 8 ROC curve analysis. Comparison of miR-21 cut-offs determined by Caramès *et al* [257] (2.8) and by this study (1.18). AUC: area under the curve. Reproduced from *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma, Front Oncol 2020 Oct 27; 10:577653*.

Simple and multiple logistic regressions were used to correlate each variable with the outcome response after CRT: “pathological complete response (TRG 0)” or “pathological incomplete response (TRG 1, 2 and 3)”. For continuous variables, linearity of the logit in the predictor was assessed using a cubic spline and Wald test of linearity.

The association between high and low miR-21 expression and clinical characteristics was tested with Chi-square test. Only variables with p value ≤ 0.25 in simple logistic regression or considered clinically relevant were selected to multiple logistic regression. Multicollinearity was also analysed through the observation of variance inflation factors. A stepwise both-selection technique was used to create the multiple regression models. ROC curve was computed and the respective AUC was calculated to assess discriminatory ability of the model. There was professional statistical review performed in this manuscript.

RESULTS

Patient clinical parameters

Demographic and clinical parameters of the 91 patients are summarized in Table 3. With 4 patients lost (4.4%), median follow up was 4.2 years.

Table 3 Patient clinical parameters

Clinical parameters		Patients (n = 91)
Gender, n (%)	Male	60 (66)
	Female	31 (34)
Age, median		68 (45 – 83)
BMI, median		26 (15 - 45)
ASA score, n (%)	Not discriminated	11 (12)
	I	2 (2)
	II	56 (62)
	III	21 (23)
	IV	1 (1)
Grade	G1/G2	85 (93)
	G3/G4	6 (7)
Location (%)	1/3 superior	19 (21)
	1/3 medium	28 (31)
	1/3 inferior	44 (48)
Tumour extension (mm), median		58 (5 - 120)
Distance to anal verge (mm), median		60 (0 - 130)
cT	1	1 (1)
	2	10 (11)
	3	64 (70)
	4	16 (18)
cN	0	9 (10)
	+	82 (90)
cM	0	78 (86)
	1	13 (14)
CRM, n (%)	Free	67 (74)
	Threatened or invaded	24 (26)
EMVI, n (%)	Negative	86 (95)
	Present	5 (5)
c Stage, n (%)	I	3 (3)
	II	8 (9)
	III	68 (75)
	IV	12 (13)
CEA (mg/mL)		1.9 (0.5 - 163)
Chemotherapy	Capecitabine based	83 (91)
	5-FU based	8 (9)
TRG (CAP), n (%)	0	15 (17)
	1	24 (26)
	2	33 (36)
	3	19 (21)

BMI Body Mass Index, ASA American Society of Anesthesiologists, CRM circumferential resection margin, EMVI extramural vascular invasion, CEA carcinoembrinonary antigen, TRG tumour regression grade, CAP College of American Pathologists.

miRNA expression in complete and incomplete responders

miRNA expression profiles were analysed in non-neoplastic and tumour rectal tissue before and after CRT in all 91 patients. Significant changes were observed when comparing incomplete and complete responders (Figure 9).

In incomplete responders, miR-21 revealed higher expression in pre-CRT tumour tissue in comparison with non-neoplastic tissue ($p = 0.03$). Post-CRT samples also presented higher levels of miR-21 in tumour tissue ($p = 0.008$). In contrast, in complete responders, miR-21 showed similar levels in pre-CRT tumour and non-neoplastic tissue.

miR-135b presented a profile equivalent to miR-21. In incomplete responders, miR-135b up-regulation was detected in tumour tissue, either pre- or post-CRT ($p < 0.0001$), whereas in complete responders equal levels were found in pre-CRT tumour samples and non-neoplastic tissue.

Although miR-145 expression showed significant differences among pre- and post-CRT non-neoplastic and tumour tissues ($p < 0.0001$) in incomplete responders, similar results were detected in complete responders, suggesting a lack of discriminative value of this miRNA. Moreover, there were no significant differences in miR-16 and miR-335 expression between groups. Thus, these results suggest that miR-21 and miR-135b might be useful biomarkers to predict treatment response.

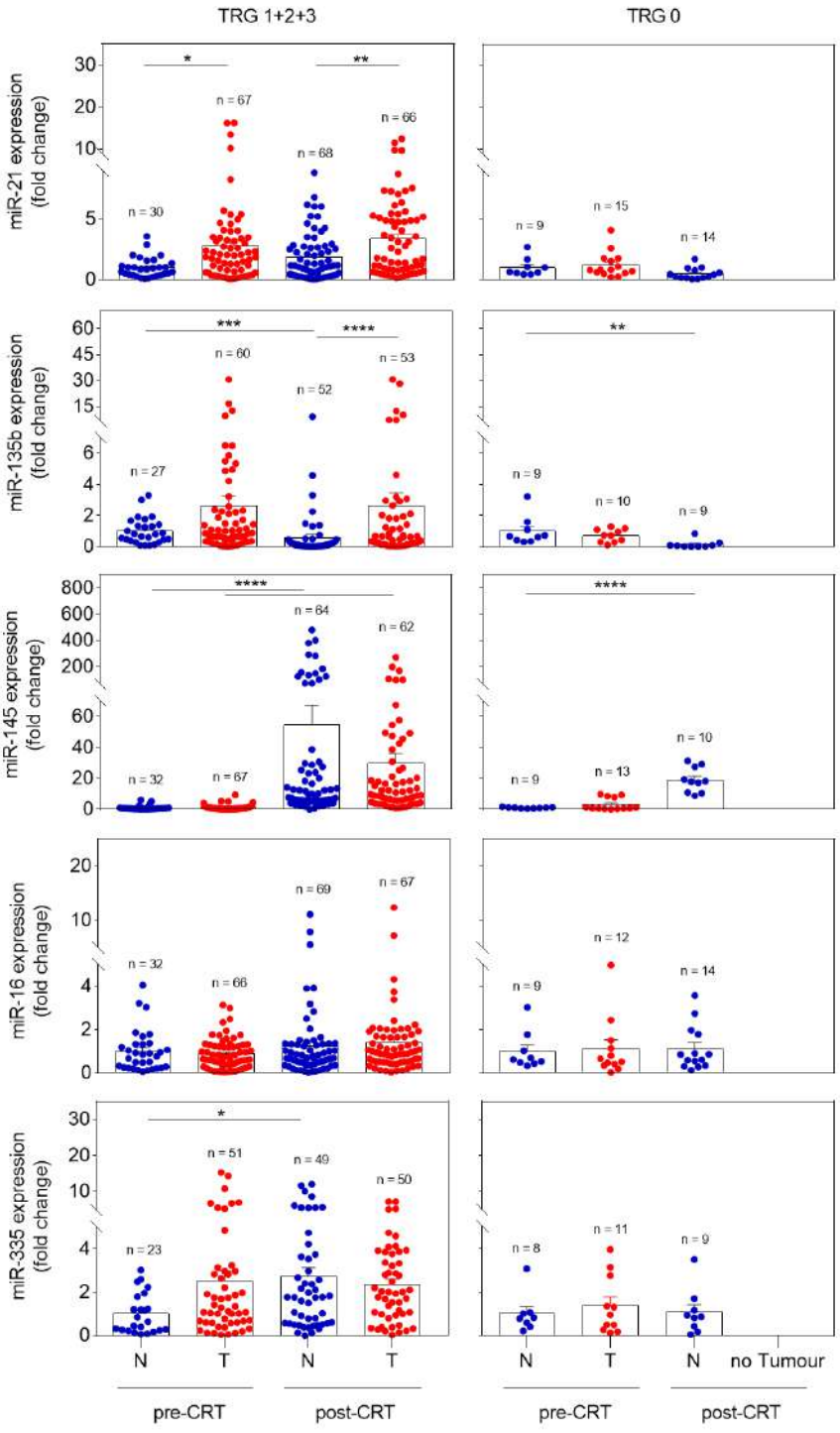

Figure 9 Expression profile of miR-21, miR-135b, miR-145, miR-16, and miR-335 in pre- and post-CRT non-neoplastic and tumour tissues in incomplete (TRG 1 +2 + 3) and complete responders (TRG 0). Pre-CRT non-neoplastic tissue samples used in this study were derived from a maximum of 37 and 10 patients in TRG 1 +2 + 3 and TRG 0 groups, respectively. Pre-CRT tumour tissue and post-CRT tissue samples were analysed from a maximum of 76 patients (TRG 1 + 2 + 3) and 15 patients (TRG 0). Data are mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), in which N corresponds to non-neoplastic tissue and T to tumour tissue. Reproduced from *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma, Front Oncol 2020 Oct 27; 10:577653.*

Identification of miRNAs involved in TRG

The significantly different expression of miRNAs between incomplete (TRG 1, 2 and 3) and complete responders (TRG 0) suggested a possible association between miRNA expression and treatment response. The relation between miRNA in pre-CRT samples and response was analysed with logistic regression (Table 4). A significant association was found between miR-21 in pre-CRT tumour tissue and TRG. Patients with expression higher than 1.18 (fold change) were 3.58 more likely to obtain an incomplete response than those with expression lower than 1.18 (p = 0.03). However, there was no association between pre-CRT non-neoplastic or tumour tissue expression of miR-135b and TRG. The same was found for miR-16, miR-145 and miR-335. Given the association of miR-21 and response, we proceeded with the study of this miRNA.

Table 4 Association between miRNA expression and TRG

Variables		OR	95 % CI	p-value
miR-21	≤0.66	1.00		
pre-CRT non-neoplastic	>0.66	1.428	0.32 - 6.79	0.6407
miR-21	≤1.18	1.00		
pre-CRT tumour	>1.18	3.58	1.13 - 12.65	0.0346
miR-135b	≤0.8	1.00		
pre-CRT non-neoplastic	>0.8	1.85	0.40 - 10.27	0.4420
miR-135b	≤1.01	1.00		
pre-CRT tumour	>1.01	2.33	0.58 - 11.62	0.25
miR-145	≤1.28	1.00		
pre-CRT non-neoplastic	>1.28	0.65	0.11 - 5.18	0.643
miR-145	≤0.73	1.00		
pre-CRT tumour	>0.73	0.88	0.26 - 3.02	0.838
miR-16	≤0.77	1.00		
pre-CRT non-neoplastic	>0.77	2.00	0.44 - 10.80	0.3806
miR-16	≤0.54	1.00		
pre-CRT tumour	>0.54	1.75	0.49 - 6.19	0.375
miR-335	≤1.16	1.00		
pre-CRT non-neoplastic	>1.16	4.5	0.64 – 91.58	0.191
miR-335	≤1.01	1.00		
pre-CRT tumour	>1.01	1.86	0.49 – 7.24	0.354

Simple logistic regression using miRNA dichotomized according to cut-offs determined with ROC curve analysis. OR: odds ratio of incomplete/non-response; CI: confidence interval.

Clinical parameters and TRG in miR-21 expressing patients

From the initial group of 91 patients, only 82 patients expressed miR-21 due to lack of amplification. An association was found between type of radio-sensitizing agent and TRG with patients treated with 5-FU presenting reduced OR of incomplete response compared with patients treated with capecitabine (OR 0.19; 95% (CI) 0.03 - 1.12, p = 0.05). A definitive trend towards reduced odds of incomplete response was also recognized with longer waiting times (OR 0.87; 95% CI 0.73 - 1.01, p = 0.08). However, there was no association between patient gender, age, weigh, ASA score, BMI, tumour location, tumour extension, histological grade, pre-therapeutic CEA, radiological involvement of the CRM, presence of EMVI, mesorectal deposits (N1c), extramesorectal nodes, cT, cN, cM, stage (TNM, AJCC) and TRG (Table 5).

Clinical parameters and levels of miR-21 expression

Although no statistically significant association between clinical parameters and expression of miR-21 was observed, a near significant association was established between this miRNA and TRG, with higher proportion of incomplete response in patients with higher miR-21 levels (p = 0.06) (Table 6). In multivariate analysis, after adjustment for clinically and statistically relevant variables, this association was again demonstrated with the odds of incomplete response 3.67 times greater in individuals with a miR-21 overexpression (> 1.18 fold change) when compared with those with lower miR-21 levels (≤ 1.18 fold change) (OR 3.67, 95% CI 1.13 - 13.5; p = 0.04) (Table 7).

Table 5 Clinical parameters and TRG in miR-21 expressing patients

Simple Logistic Regression		TRG 0 n = 15	TRG 1+2+3 n = 67	OR	95 % CI	p-value
Continuous Variables		Median (Max-Min)	Median (Max-Min)			
Age		67.0 (53 - 81)	68 (45.0 - 83)	1.00	0.94 - 1.06	0.976
Weight		70.0 (45 - 113)	68 (44.0 - 119)	0.99	0.96 - 1.03	0.645
BMI		25.0 (19 - 41)	26 (15.0 - 45)	1.00	0.91 - 1.13	0.921
Tumour extension (mm)		54.5 (21 - 110)	56 (5 - 120)	0.99	0.97 - 1.03	0.901
CEA		2.8 (0.5 - 8.3)	1.9 (0.5 - 163)	1.07	0.99 - 1.29	0.299
Weeks Post Chemo		11 (7.0 - 28)	10 (2.0 - 21)	0.87	0.73 - 1.01	0.081
Categorical Variables		Number	Number			
Gender	Male	11	45	1.00		
	Female	4	22	1.34	0.41 - 5.29	0.643
Tumour Location	0	3	14	1.00		
	1	8	16	0.43	0.08 - 1.81	0.271
	2	4	37	1.98	0.35 - 10.13	0.407
ASA	1+2	9	54			
	3+4	6	13	0.36	0.11 - 1.24	0.0955
CRM MR	Free	11	50		1.00	
	Threatened	1	4	0.88	0.12 - 18.11	0.913
	Invaded	3	13	0.95	0.25 - 4.66	0.947
Extramesorectal	Negative	12	43	1.00		
nodes	Positive	3	24	2.23	0.63 - 10.50	0.247
cT	1+2	1	8	1.00		
	3+4	14	59	0.53	0.03 - 3.23	0.561
cN	0	2	6	1.00		
	1	13	61	1.56	0.21 - 7.721	0.608
cM	0	14	57	1.00		
	1	1	10	2.46	0.42 - 46.96	0.41
Stage	I	1	2	1.00		
	II	2	5	1.25	0.04 - 23.53	0.880
	III	11	51	2.32	0.10 - 26.38	0.508
	IV	1	9	4.50	0.14 - 156.82	0.352
Stage	I + II	3	7	1.00		
	III + IV	12	60	2.14	0.42 - 8.99	0.315
Chemotherapy	Capecitabine	12	64	1.00		
	5-FU	3	3	0.188	0.03 - 1.12	0.05

Simple logistic regression analysis using TRG as dependent variable and clinical/ molecular variables as independent variables. From the initial group of 91 patients, 82 expressed miR-21. TRG Tumour regression grade, OR odds ratio of incomplete response, CI confidence interval, BMI body mass index, CEA, carcinoembryonic antigen, ASA American Society of Anaesthesiologists, CRM circumferential resection margin, MR magnetic resonance.

Table 6 Clinical parameters and levels of miR-21 expression

Variables		Number (%)	High miR-21	Low miR-21	p-value
miR-21 pre-CRT tumour		82 (100)	48 (58.5)	34 (41.5)	
Age	<60	15 (18.3)	7 (14.6)	8 (23.5)	0.302
	≥60	67 (81.7)	41 (85.4)	26 (76.5)	
Sex	Male	56 (68.3)	32 (66.7)	24 (70.6)	0.707
	Female	26 (31.7)	16 (33.3)	10 (29.4)	
BMI	Low weight	1 (1.2)	0 (0)	1 (2.9)	0.236
	Normal	27 (32.9)	17 (35.4)	10 (29.4)	
	Pre-obesity	39 (47.6)	25 (52.1)	14 (41.2)	
	Obesity	15 (18.3)	6 (12.5)	9 (26.5)	
ASA score	1	2 (2.4)	1 (2.1)	1 (2.9)	0.330
	2	53 (64.6)	29 (60.4)	24 (70.6)	
	3	18 (22)	11 (22.9)	7 (20.6)	
	4	1 (1.2)	1 (2.9)	0 (0)	
	ND	8 (9.8)	7 (14.6)	1 (2.9)	
Stage pre-CRT	I	3 (3.7)	1 (2.1)	2 (5.9)	0.720
	II	7 (8.5)	4 (8.3)	3 (8.8)	
	III	62 (75.6)	36 (75.0)	26 (76.5)	
	IV	10 (12.2)	7 (14.6)	3 (8.8)	
Stage post-CRT	0	12 (14.6)	6 (12.5)	6 (17.6)	0.607
	I	6 (7.3)	4 (8.3)	2 (5.9)	
	II	6 (7.3)	5 (10.4)	1 (2.9)	
	III	9 (11.0)	4 (8.3)	5 (14.7)	
	IV	3 (3.7)	1 (2.1)	2 (5.9)	
	NA	5 (6.1)	4 (8.3)	1 (2.9)	
	ND	41 (50)	24 (50.0)	17 (50.0)	
Grade pre-CRT	Low	77 (93.9)	45 (93.8)	32 (94.1)	1.00
	High	5 (6.1)	3 (6.2)	2 (5.9)	
cT	1	1 (1.2)	1 (2.1)	0 (0.0)	0.852
	2	8 (9.8)	5 (10.4)	3 (8.8)	
	3	59 (72.0)	34 (70.8)	25 (73.5)	
	4	14 (17.1)	8 (16.7)	6 (17.6)	
cN	0	8 (9.8)	4 (8.3)	4 (11.8)	0.606
	1	74 (90.2)	44 (91.7)	30 (88.2)	
cM	0	71 (86.6)	41 (85.4)	30 (88.2)	0.712
	1	11 (13.4)	7 (14.6)	4 (11.8)	
pTRG	TRG 0	15 (18.3)	5 (10.4)	10 (29.4)	0.064
	TRG 1	21 (25.6)	16 (33.3)	5 (14.7)	
	TRG 2	32 (39.0)	20 (41.7)	12 (35.3)	
	TRG 3	14 (17.1)	7 (14.6)	7 (20.6)	
Distant recurrence	No	60 (73.2)	33 (68.8)	27 (79.4)	0.283
	Yes	22 (26.8)	15(31.2)	7 (20.6)	
Local recurrence	No	75 (91.5)	43 (89.6)	32 (94.1)	0.694

	Yes	7 (8.5)	5 (10.4)	2 (5.9)	
Death	No	61 (74.4)	33 (68.8)	28 (82.4)	0.164
	Yes	21 (25.6)	15 (31.2)	6 (17.6)	

Table 7 Association between clinical parameters and TRG

Variables		OR	95 % CI	p-value
Stage	1+2	1.00		
	3+4	2.16	0.388 - 10.16	0.341
miR-21	≤1.18	1.00		
	>1.18	3.67	1.126 - 13.49	0.036
ASA score	1+2	1.00		
	3+4	0.33	0.090 - 1.185	0.082

Multiple logistic regression analysis using TRG as dependent variable and disease stage, miR-21 and ASA score as independent variables. OR odds ratio, CI confidence interval, ASA American Society of Anaesthesiologists.

miR-21 expression and oncological outcomes

Overall survival (OS) at 2 and 5 years was 90% (95% CI 83.4 - 96.9) and 72% (95% CI 61.6 - 85.1), respectively. Overall disease free survival (DFS) at 2 and 5 years was 74.1% (95% CI 64.4 - 84.8) and 66% (95% CI 55 - 80), respectively (Figure 10).

Overall survival was not influenced by age, gender, tumour location, grade, mesorectal nodes, extramesorectal nodes, type of radio-sensitizing agent, post-operative complications and levels of miR-21 ($p = 0.36$) (Figure 11 and Figure 12). As expected, there was an impact in OS in relation to T ($p < 0.0001$) mesorectal tumour deposits, N1c ($p = 0.003$), distant metastasis M ($p = 0.002$), stage ($p = 0.0004$) and TRG ($p = 0.04$) with a borderline significance for threatened CRM ($p = 0.05$) (Figure 11). Also, there was increase death risk in individuals with higher cT (HR = 4.78; 95% CI 1.96 - 11.66, $p = 0.0006$), higher stage (HR = 11.1; 95% CI 1.34 - 91.88, $p = 0.03$), threatened CRM (HR = 4.24; 95% CI 1.19 - 15.08, $p = 0.03$), positive N1c (HR = 5.47; 95% CI 1.56 - 19.14, $p = 0.008$), distant metastasis (HR = 3.78; 95% CI 1.52- 9.4, $p = 0.004$) and TRG 3 (HR = 3.25; 95% CI 0.83 - 12.71, $p = 0.08$) (Table 8). No association was, however, established between miR-21 expression and risk of death (Table 8).

Finally, the utility of miR-21 as a predictor of survival was investigated and the model of prediction, in multivariate analysis, adjusted to the most relevant clinical variables, showed a close to significant association between risk of death and higher miR-21 expression (HR = 2.68; 95% CI 0.86 - 8.36, $p = 0.09$) (Table 9).

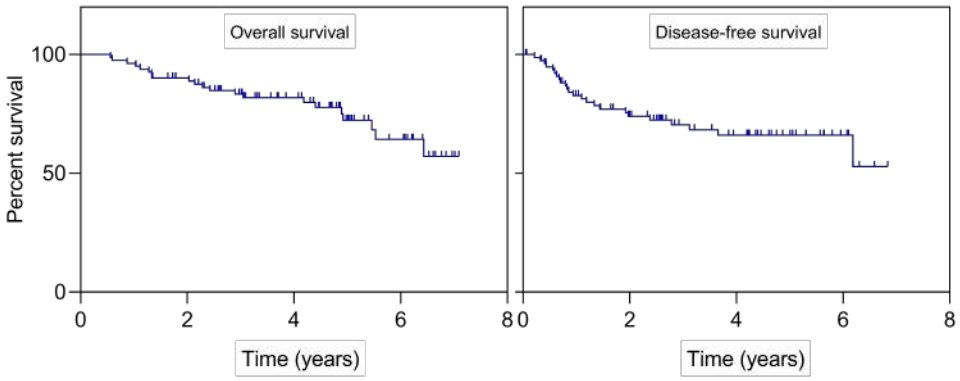

Figure 10 Patient outcomes in miR-21-expressing patients. Kaplan–Meier curves for overall survival and disease-free survival. Reproduced from *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma, Front Oncol 2020 Oct 27; 10:577653.*

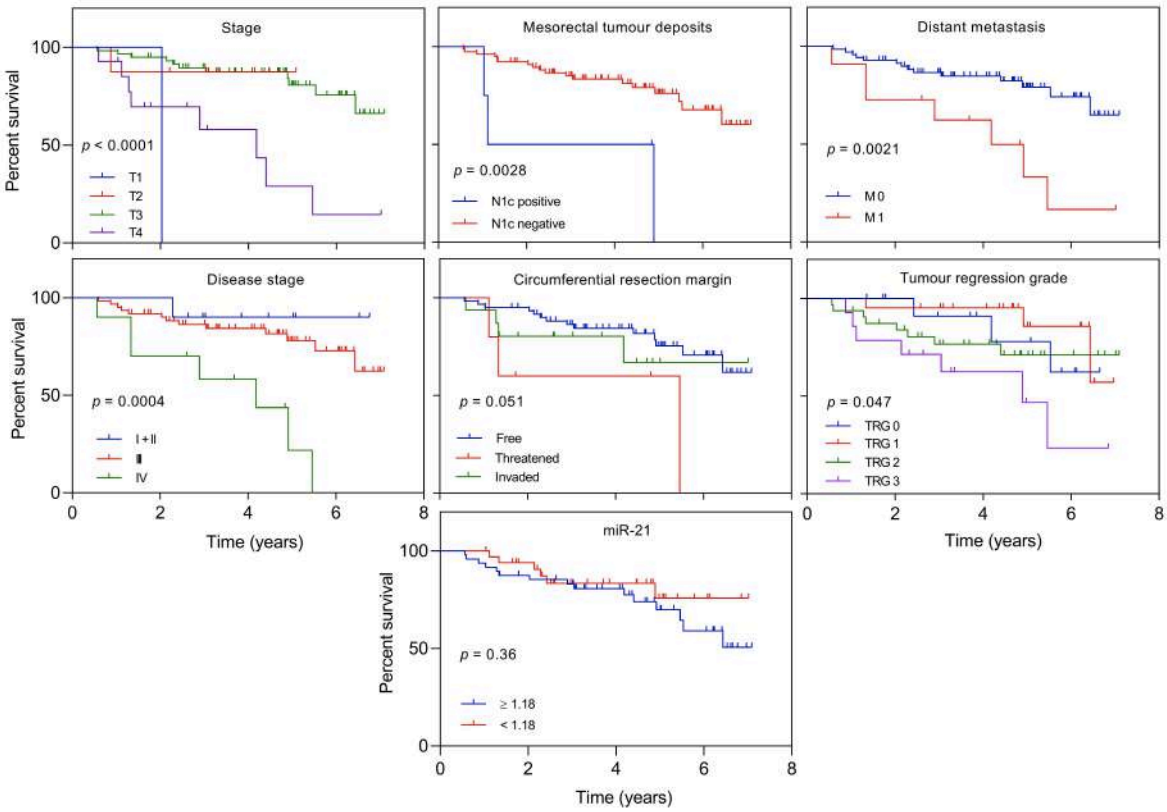

Figure 11 Overall survival according to clinical and oncological parameters. Kaplan–Meier curves estimating overall survival according to stage, mesorectal tumour deposits (cN1c), M, stage, circumferential resection margin (CRM) involvement, tumour regression grade and levels of miR-21. Reproduced from *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma, Front Oncol 2020 Oct 27; 10:577653.*

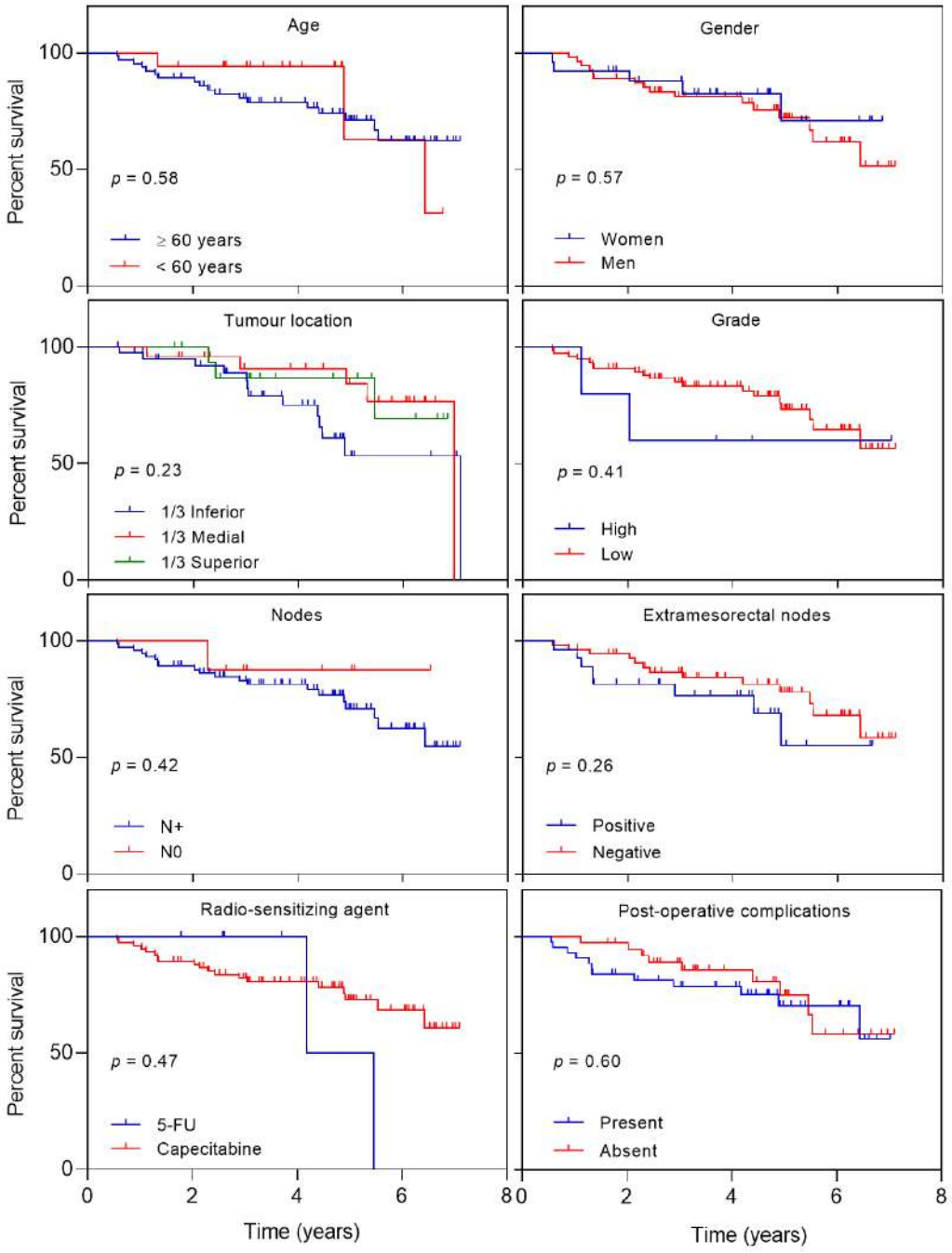

Figure 12 Overall survival according to clinical and oncological parameters. Kaplan-Meier curves estimating overall survival according to age, gender, tumour location, grade, nodes, extramesorectal nodes, type of radio-sensitizing agent and post-operative morbidity. Reproduced from *Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma, Front Oncol 2020 Oct 27; 10:577653*

Table 8 Patient survival according to miR-21 expression and clinical parameters

		Patients n = 82	Deaths n = 21	Survival		Simple Cox Proportional Hazards Models			
				Mean	p value	Coef	HR	95 % CI	p value
miR-21	<1.18	34	6	6.04			1.00		0.36
	≥1.18	48	15	5.50	0.36	0.44	1.56	0.60 - 4.03	
Age	<60	17	3	5.81	0.58		1.00		0.57
	>60	65	18	5.51		0.35	1.42	0.41 - 4.8	
Sex	Male	56	16	5.56	0.57		1.00		0.57
	Female	26	5	5.82		-0.29	0.75	0.27 - 2.04	
Tumour location	1/3 upper	17	3	6.09	0.14		1.00		
	1/3 middle	24	5	6.13		0.05	1.045	0.25 - 4.40	0.94
	1/3 lower	41	13	5.16		0.91	2.49	0.70 - 8.85	0.158
ASA score	1+2	55	14	5.71	0.97		1.00		
	3+4	19	5	5.44		0.10	1.11	0.39 - 3.094	0.879
	ND	8	2	5.10		0.12	1.12	0.25 - 4.99	0.986
Stage	I+II	10	1	6.32	0.0004		1.00		
	III	61	13	5.74		0.83	2.31	0.30 - 17.65	0.4218
	IV	11	7	3.54		2.41	11.10	1.34 - 91.88	0.0256
Grade	Low	77	19	5.74	0.41		1.00		
	High	5	2	4.87		0.60	1.83	0.42 - 7.88	0.42
CRM	Free	61	14	5.91	0.051		1.00		
	Threatened	5	3	3.77		1.45	4.24	1.19 - 15.08	0.025
	Invaded	16	4	5.47		0.51	1.67	0.54 - 5.142	0.37
EMVI	Negative	77	20	4.45	0.77		1.00		0.768
	Positive	5	1	4.20		0.31	1.36	0.17 - 10.41	
N1c	Negative	78	18	5.15	0.0028		1.00		0.00788
	Positive	4	3	2.98		1.69	5.47	1.56 - 19.14	
Extramesorectal nodes	Negative	55	13	5.77	0.26		1.00		
	Positive	27	8	5.15		0.51	1.67	0.68 - 4.07	0.263
cT	T1-3	68	13	6.05	0,0001		1.00		
	T4	14	8	3.73		1.56	4.78	1.96 - 11.66	0.0006
cN	0	8	1	6.25	0.42		1.00		
	1	74	20	4.48		0.81	2.24	0.29 - 16.7	0.432
cM	0	71	14	5.98	0.0021		1.00		
	1	11	7	4.02		1.33	3.78	1.52 - 9.4	0.00416
TRG	0	15	3	5.94	0.047		1.00		
	1	21	3	6.32		0.49	0.61	0.12 - 3.05	0.5504
	2	32	8	5.54		0.34	1.41	0.37 - 5.35	0.6130
	3	14	7	4.31		1.18	3.25	0.83 - 12.71	0.0897
Chemotherapy	Capecitabine	76	19	5.24	0.47		1.00		
	5-FU	6	2	4.83		0.54	1.71	0.39 - 7.43	0.476
Post Op Complications	Negative	38	9	5.85	0.6		1.00		
	Positive	44	12	5.55		0.23	1.26	0.53 - 0.98	0.604

Table 9 Association between patient survival and miR-21 expression.

		Multiple Cox Proportional Hazards Models				Multiple Cox Proportional Hazards Models			
		Coef	HR	95 % CI	p value	Coef	HR	95 % CI	p value
miR-21	< 1.18	Not Included					1.00		
	≥ 1.18					0.99	2.68	0.86 - 8.36	0.089
Mesorectal deposits	Negative		1.00				1.00		
	Positive	1.84	6.26	1.74 - 22.48	0.005	2.49	12.17	2.61 - 56.70	0.001
cT	T1-3		1.00				1.00		
	T4	1.63	5.09	2.06 - 12.61	0.0004	1.69	5.45	2.17 - 13.63	0.0003
C-Statistics		0.671				0.674			

Multiple Cox Proportional Hazards Models obtained with stepwise variable selection. HR: hazard ratios; CI: confidence interval.

DISCUSSION

RC patients treated with CRT urgently need biomarkers to distinguish responders from non-responders and avoid overtreatment, surgery delays and toxicities. Prediction of response would allow individualized treatment, with non-responders avoiding neoadjuvant therapy and complete responders eluding mutilating resections.

miRNAs, known to regulate several oncogenic CRC pathways, have been viewed as promising biomarkers. In this work we investigated if miRNAs could be used as biomarkers to predict response to CRT by analysing their expression in incomplete and complete responders.

miR-145 and miR-335 are acknowledged to act as tumour suppressors [258][249] and miR-145 is overexpressed in post- CRT tumour tissue in comparison with pre-CRT with significant correlation with tumour regression [259]. In our work, no differences were detected in these miRNAs before and after CRT and no correlation was found with response. In addition, miR-16 has been described as a tumour suppressor with downregulation predicting poor prognosis in CRC [224]. In our study, miR-16 was not a predictor of response either. miR-135b is an oncomiR that often mediates CRC genes whose overexpression has been correlated with tumour stage and poor clinical outcome [242]. We have further analysed its potential as predictor of response to CRT and found significant differences in expression. In incomplete responders, higher miR-135b levels were found in both pre- and post-CRT tumour tissues comparing with non-neoplastic tissues, whereas in complete responders similar expression was obtained in all samples. We could not, however, correlate miR-135b expression with clinical parameters or TRG.

Finally, in our study we found that incomplete responders had higher miR-21 expression in tumour tissue in comparison with non-neoplastic tissue in both pre- and post-CRT samples. In contrast, complete

responders had similar levels in all samples. Moreover, a significant association was discovered between pre-CRT tumour miR-21 levels and TRG, with a 3.67 odds of non-response in patients with expression higher than 1.18 ($p = 0.04$). Higher miR-21 expression in the tumour prior to treatment was indicative of a worst response. As expected, OS was influenced by cT, cM, N1c and but no association was noted between risk of death and miR-21 expression. Patients with miR-21 overexpression exhibited less response to standard CRT dose and expression levels before neoadjuvant therapy had the potential to predict response. This did not, however, translate in a change in survival.

miR-21 is often up-regulated in solid tumours influencing cell proliferation, invasion and apoptosis [230]. Considered to be an oncomiR, multiple studies report its role in CRC biology as a screening, diagnostic and prognostic biomarker [198],[201],[202],[226],[260]. Also, miR-21 up-regulation has been related to advanced stage, presence of positive LN, venous invasion and metastatic behaviour [228],[229].

In contrast to colonic cancer, very limited data is available on miRNA expression and response to CRT in RC with most patients treated with 5-FU based therapies and not capecitabine [261][262][263]. So far, miR-21 has been described to induce resistance to 5-FU [264], which could be a potential explanation for the 5-FU-based CRT response.

Literature is controversial regarding the use of miR-21 as biomarker of response in RC [261],[262]. [265]. In one study with 76 RC biopsies, high pre-CRT miR-21 could discriminate responders from non-responders with an OR of 9.75 (95% CI 2.24 to 42) [257]. Recently, 96 complete responders had significantly inferior miR-21 expression comparing with patients with incomplete response ($p = 0.01$), with an AUC of 0.669 (95% CI 0.55 - 0.79, $p = 0.01$) [266]. These observations are in accordance with our own results and with the well-reported miR-21 oncomiR function. Contrarily, in another study, RC patients treated with 5-FU based CRT had higher miR-21 in post-CRT tumour tissue than in pre-CRT tumour and post CRT normal tissues [259]. It has also been reported overexpression of miR-21 in patients with complete response [267][268]. It is important to note, however, that in one of these studies [268], the responders group involved a different set of patients, including individuals submitted to surgery with pathological complete response (pCR) and patients with complete clinical response (cCR) not treated with surgery but only observed by follow up [268]. The latest might have had undetectable residual disease and not be a real pCR. This different response assessment invalidates an accurate comparison of results and may explain the distinct observations when compared with our work.

Overall, the heterogeneity of results is related to the fact that most published studies included patients with colon and RC, two distinct entities with different treatment strategies that previous contributions failed to separate. Patient variability, nature of biological samples (blood, tissue, serum or faeces), miRNA extraction, array platforms, bioinformatics analysis and different TRG grading systems also contribute to these discrepancies. Likewise, it is possible that different miRNA signatures are present within a population and transcriptome varies according to tumour site.

In this study we recognized the significance of miR-21 expression in RC in response to neoadjuvant CRT. Although including a sizeable group of patients with uniform sampling and treatment, there is a potential for intratumoral heterogeneity. If confirmed as a biomarker, translation to clinical practice with miR-21 inclusion in treatment algorithms may allow a stratification of responders and better selection of candidates for CRT.

Original Paper 3

Evaluation of Tissue and Circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer

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Abstract

***Background**: As response to chemoradiotherapy in patients with locally advanced rectal cancer is variable it is urgent to find predictive biomarkers of response.*
***Objectives**: We investigated miR-21 as tissue and plasma biomarker of response to CRT in a prospective cohort of RC patients.*
***Methods**: The expression of miR-21 was analysed in pre- and post-CRT rectal tissue and plasma in 37 patients with RC. Two groups were defined: pathological responders (TRG 0, 1 and 2) and non-responders (TRG 3). The association between miR-21, clinical and oncological outcomes was assessed.*
***Results**: miR-21 was upregulated in tumour tissue and we found increased odds of overexpression in pre-CRT tumour tissue (OR: 1.63; 95% CI: 0.40–6.63, p = 0.498) and pre-CRT plasma (OR: 1.79; 95% CI: 0.45–7.19, p = 0.414) of non-responders. The overall recurrence risk increased with miR-21 overexpression in pre-CRT tumour tissue (HR: 2.175, p = 0.37);*
***Conclusions**: Significantly higher miR-21 expression is observed in tumour tissue comparing with non-neoplastic. Increased odds of non-response are reported in patients expressing higher miR-21, although without statistical significance. This is one of the first studies on circulating miR-21 as a potential biomarker of response to CRT in RC patients.*

Keywords: biomarkers; miR-21; chemoradiotherapy; rectal cancer; tumour regression grade

RATIONAL AND AIMS

Rational: miRNAs associated with RC have been identified in tumour tissue, however, the need for a non-invasive prediction tool prompted their investigation in serum and plasma as circulating markers. The previous study by our Research Group identified an association between miR-21 expression in pre-CRT rectal tumour tissue and TRG, with higher levels correlating with worse pathological response.

So far, no studies have investigated the potential of circulating miR-21 as molecular predictor of response to neoadjuvant CRT. This motivated the validation of our prior results in a prospective cohort of patients and the investigation of this miRNA in blood. This study hypothesised that the expression of specific miRNAs in RC plasma could be associated with response to CRT, being able to predict it.

Aims: Using a prospective group of patients with RC, we investigated the relation between tissue and plasma miR-21 and evaluated its potential use as a tissue and circulating biomarker of response to CRT. The association between miR-21 and clinical and oncological outcomes was also assessed.

miRNA associated with CRC have been identified in tumour tissue and also in plasma and serum. It seems that CRC-derived miRNAs are released into the blood stream so plasma miRNA expression may reflect the signature of the tumour tissue. miRNA released into systemic circulation, freely or in exosomal shells, is less vulnerable to RNase-mediated degradation, remaining remarkably stable, reproducible and not degraded for a long half-life. The idea of a correlation between circulating and tissue miRNA supports the hypothesis that plasmatic miRNAs can serve as biomarkers of disease or disease response to therapy.

Direct identification of circulating miRNAs as liquid biopsies can provide information for diagnosis, prognosis and predictive responses to treatment in CRC without the need for tumour-tissue biopsies. This particular characteristic of miRNAs is also a central reason for their study as biomarkers.

MATERIALS AND METHODS

This was a prospective observational study. Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institutional Human Research Committee and Ethical Committee. The study was registered in the Portuguese Data Protection Agency.

Patients and Tissue Samples

A total of 37 patients diagnosed with RC between April 2017 and June 2019 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal) treated with long course CRT and proctectomy were eligible. Patients had a preoperative staging with pelvic MR, thoraco-abdominal CT and EAUS when pelvic MR was not clinically possible.

Histopathological features were confirmed by pathological analysis and patients were staged according to TNM staging system (8th edition, 2017). Patients with other histological types of rectal malignancy, not submitted to CRT or surgical resection, pregnant or under the age of 18 were excluded.

Fresh frozen tissue samples were collected before and after CRT, during pre-therapeutic colonoscopy and from the protectomy specimen, respectively. Pre-CRT rectal tumour biopsies were gathered from all patients but post-CRT tumour tissues were available only from patients without a pathological complete response (Fig.13). To allow a direct comparison of rectal cancer to matched non-neoplastic rectal mucosa, we collected corresponding adjacent (>1 cm distant) non-tumour tissue both in biopsies and protectomy specimens. Retrieved tumour and non-neoplastic tissue underwent histological confirmation by a pathologist. A fixed amount of tissue (80 micron) was extracted across samples, immediately frozen with CO2 prior to storage at - 80 ºC.

In addition, liquid biopsies (plasma) were also collected from patients, before and after CRT, at the time of pre-treatment staging colonoscopy and 24 hours after proctectomy. Peripheral blood was collected in vacutainer liquid EDTA 6-mL blood collection tubes and peripheral blood cells and plasma were separated by density gradient separation. Plasma was then stored and frozen at - 80 ºC until RNA extraction.

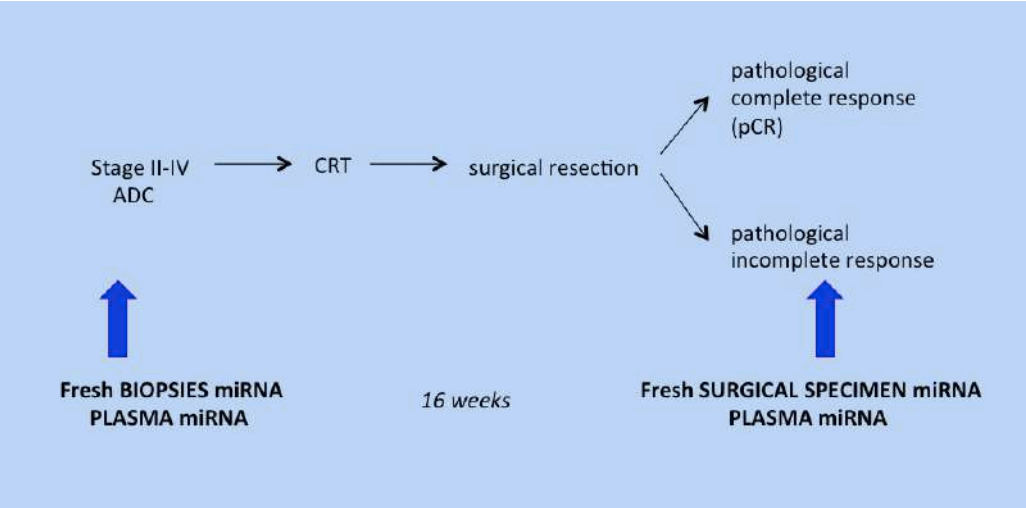

Figure 13 Timings of tissue and plasma sampling

Neoadjuvant Treatment

All patients underwent neoadjuvant CRT that consisted of a total dose of 50.4 Gy of pelvic irradiation, 5 times a week, with a daily fraction of 2 Gy using at least a four-field technique. Radiation was delivered with capecitabine (825 mg/m2/twice daily, continuously during RT) or 5-fluoruocil (5-FU) (225 mg/m2 continuous infusion during RT. Surgery was performed 10–12 weeks after CRT.

Assessment of Pathological Response

Pathology specimens were graded by TRG according to the CAP guidelines. Two independent pathologists blinded to patient clinical data evaluated TRG categorizing tumours in: TRG 0 or complete response (no viable tumour cells), TRG 1 or moderate score (single cells or little groups of cancer cells), TRG 2 or minimal response (residual cancer outgrown by fibrosis), TRG 3 or poor response (minimal or no tumour killing with extensive residual cancer) (Table 10). Two groups of patients were defined: responders (TRG 0, 1, and 2) composed of a total of 21 patients and non-responders (TRG 3) composed of a total of 16 patients.

Table 10 CAP TRG Score.

CAP TRG Score	
0	No viable residual tumour (complete response: pCR)
1	Marked response (minimal residual cancer with single cells or small groups of cancer cells)
2	Moderate response (residual cancer outgrown by fibrosis)
3	Poor or no response (extensive residual cancer)

Follow up

Patients had a median of 603 (196–1007) days of follow up with no patients lost.

RNA Isolation from Fresh Frozen Tissues and Serum

Total RNA was extracted using RibozolTM reagent (VWR International, Radnor, PA, USA) in pre- and post-CRT fresh frozen non-neoplastic and tumour rectal tissues samples according to the manufacturer's instructions, whereas miRNeasy serum/plasma advanced kit (Qiagen, GmbH, Germany) was used to isolate RNA in pre- and post-CRT plasma samples from a total amount of 200 μL of plasma. In plasmatic RNA isolation, an exogenous control was added to each sample to monitor extraction efficiency and to further normalize miRNA expression data. Thus, 1.6x108 copies/μL of synthetic spike-in control Caenorhabditis elegans miR-39 5'-phosphorylated (cel-miR-39-3p_5P) was added according to the miRNeasy kit instructions. RNA extracted from tissue and serum was eluted in 50 μL and 20 μL of RNase-free water, respectively. For a better evaluation of miRNAs quantity in total RNA, the concentration of miRNA was determined using QubitTM miRNA Assay kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). All RNA samples were stored at -80ºC.

cDNA Synthesis and Real-Time PCR (RT-PCR)

cDNA synthesis was performed using TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 2 μL of total RNA (corresponding to 2 ng of RNA extracted from tissue) were extended by a 30 poly-A tailing reaction and a 50-adaptor ligation to the mature miRNAs. miRNAs were reverse transcribed into cDNA by reverse transcription using Universal RT primers. In order to improve detection of low-expressing

miRNA targets, a pre amplification of the cDNA was performed using the Universal miR-Amp Primers and miR-Amp Master Mix to uniformly increase the amount of cDNA for each target, maintaining the relative differential expression levels. cDNA samples were stored at -20ºC. Real-Time PCR was performed on a QuantstudioTM 7 Flex real-time PCR instrument (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) with TaqManTM Advanced microRNA Assays (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) to assess the expression profile of hsa-miR-21-5p (Assay ID 477975_mir). All reactions were performed in duplicate.

Since a consensual endogenous control for miRNA expression in rectal tissue has still not been determined, normalization was performed with hsa-miR-484 (Assay ID 478308_mir) for tissue miRNA expression analysis. In our previous retrospective study miR-484 was identified as the most stably expressed miRNA with the lowest expression variability when compared with mir-1228-5p, miR-345-5p, miR-103a-3p and the small nuclear (snRNA) U6 and RNU6B, considered endogenous controls for CRC tissues and/or serum. For serum miRNA expression analysis, normalization was performed with cel-mir-39-3p (Assay ID 478293_mir). Expression levels were calculated by the threshold cycle ($2^{-\Delta\Delta Ct}$ method), when amplification values were detected. Due to lack of amplification values of miRNAs detected for all tissues, a variable number of samples have been included in each tissue miRNA expression profile. To determine fold change, pre-CRT non-neoplastic tissue and pre-CRT plasma samples were used as controls in tissue and plasma expression analysis, respectively. Fold change values were calculated as the ratio between miR-21 levels in tissue or plasma and the mean of the controls' values.

Statistical Analysis

miRNA expression was analysed using the Graph Pad Prism software package, version 7.0 (GraphPad software Inc., San Diego, CA, USA). Normal distribution was determined using the D'Agostino&Pearson omnibus test. Statistical differences between patient groups in plasma expression data were evaluated by two-tailed non-parametric Mann–Whitney U test, whereas tissue expression data was analysed using the one-way analysis of variance (ANOVA) Kruskal–Wallis non-parametric Dunn's multiple comparison test. Spearman correlation coefficient was used to test the correlation between plasma and tissue miRNA expression levels (Fig 13). Using contingency tables OR were estimated and the p-value associated were obtained resorting to Fisher test. ROC curves were used to calculate optimal cut-offs for miR-21 in pre-CRT normal, tumour tissue and blood determined as the point closest to the top left part of the plot with perfect sensibility and sensitivity. miR-21 was then dichotomized according to these cut-offs (Table 11). Kaplan–Meier survival curves were compared with Log-rank test and simple Cox proportional hazards models were adjusted to analyse the association of each variable with disease free survival. Overall survival was not possible to determine in this study due to the reduced number of deaths observed (n_{death} = 3). Data was analysed with SPSS (IBM, version 20) and R (version 3.0.2). $p \leq 0.05$ acknowledged statistical significance. There was professional statistical review performed in this manuscript.

Table 11 Predictive value of miR-21 cut off.

miR-21 cut-points	Pre-CRT tumour tissue 2.61	Pre-CRT non-neoplastic tissue 1.2	Pre-CRT serum 0.54	Post-CRT serum 0.84
Sensitivity (%)	53	56	44	50
Specificity (%)	47	57	47	53
PPV (%)	53	60	50	56
NPV (%)	47	53	41	47

Cut-off derived by ROC curve. miR-21 estimated cut-points: 2.61 in pre-CRT tumour tissue; 1.2 in pre-CRT non-neoplastic tissue; 0.54 in pre-CRT serum; 0.84 in post-CRT serum. PPV: positive predictive value; NPV: negative predictive value.

RESULTS

Patient Clinical Parameters

Demographic and clinical parameters of the 37 patients are summarized in Table 12.

Table 12 Patient clinical parameters

Clinical parameters		Patients (n = 37)
Gender, n (%)	Male	25 (68)
	Female	12 (32)
Age, median		62 (42 – 88)
BMI, median		25 (20 - 35)
ASA score, n (%)	Not discriminated	3 (8)
	I	0 (0)
	II	22 (60)
	III	12 (32)
	IV	0 (0)
Tumour grade	G1/G2	29 (78)
	G3/G4	2 (6)
	Not discriminated/determinable	6 (16)
Tumour location (%)	1/3 superior	1 (3)
	1/3 medium	14 (38)
	1/3 inferior	22 (59)

Tumour extension (mm), median		55 (19 - 90)
Distance to anal verge (mm), median		50 (0 - 100)
cT	1	0 (0)
	2	7 (19)
	3	25 (68)
	4	5 (13)
cN	0	3 (8)
	+	34 (92)
cM	0	35 (95)
	1	2 (5)
CRM, n (%)	Free	17 (46)
	Threatened	4 (11)
	Invaded	16 (43)
EMVI, n (%)	Negative	25 (68)
	Present	12 (32)
c Stage, n (%)	I	0 (0)
	II	2 (5)
	III	33 (90)
	IV	2 (5)
CEA (mg/mL), median		1.7 (0.5 - 96)
CRT	5-FU based	4 (11)
	Capecitabine based	33 (90)
TRG (CAP), n (%)	0	9 (24)
	1	7 (19)
	2	5 (14)
	3	16 (43)

miR-21 Expression in Responders and Non-Responders

miRNA expression profile was analysed in non-neoplastic and tumour rectal tissues as well as in plasma, collected before and after CRT. The differences observed when comparing responders (TRG 0-2) and non-responders (TRG 3) are demonstrated in Figure 13. In responders, miR-21 revealed significantly higher expression ($p = 0.0013$) in pre-CRT tumour tissue when compared with non-neoplastic tissue.

The same expression profile was observed in post-CRT tissue samples with higher levels of miR-21 in the tumour tissue. However, this profile was also detected in non-responders with overexpression of miR-21 detected in pre-CRT ($p = 0.0004$) and post-CRT tumour tissue when compared with non-neoplastic tissue (Figure 14A).

Regarding miR-21 expression analysis in plasma (Figure 14b), a slight increase with no statistical significance was observed in post-CRT plasma miR-21 expression in responders comparing with pre-CRT samples. Again, no differences were evident before and after treatment in non-responders.

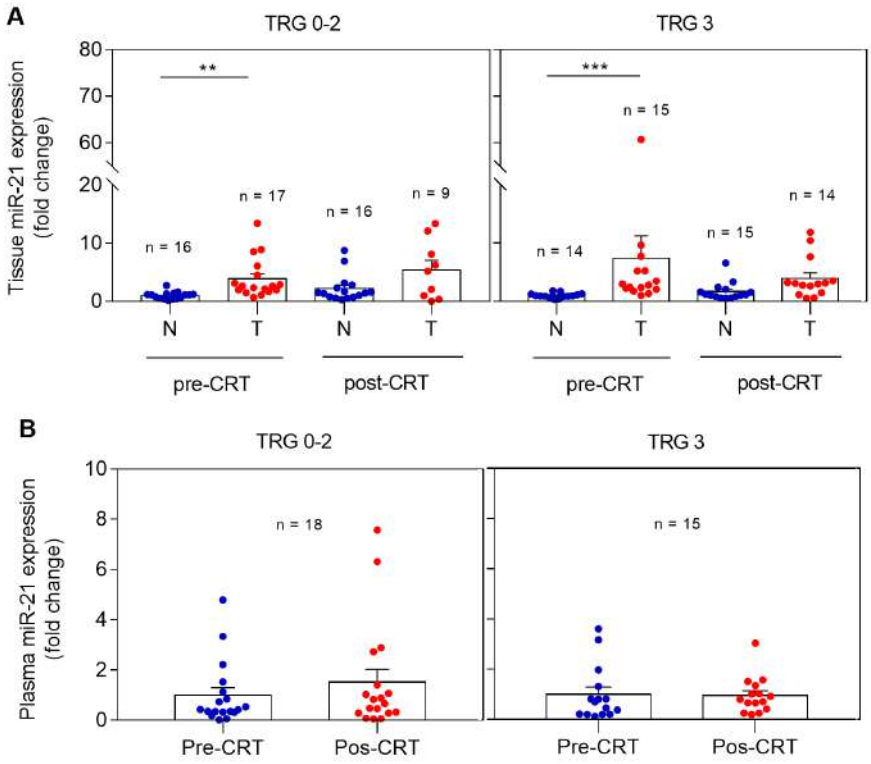

Figure 14 Expression profile of miR-21 in pre- and post-CRT samples in responders (TRG 0-2) and non-responders (TRG 3). (a) miR-21 levels in non-neoplastic and tumour tissues; (b) miR-21 levels in plasma. Fold changes in tissue and plasma miR-21 expression are calculated from pre-CRT non-neoplastic tissue and pre-CRT plasma expression, respectively. Data are mean ± SEM.N corresponds to non-neoplastic tissue and T to tumour tissue. ** $p \leq 0.01$, *** $p \leq 0.001$. Reproduced from *Evaluation of tissue and circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer, Pharmaceuticals 2020, 13, 246*

Clinical Parameters and TRG

There was no statistically significant association between clinical parameters and TRG (Table 13). Nevertheless, we observed in our sample a reduced odds of non-response (TGR 3) in women (OR: 0.54; CI: 0.13–2.27; p = 0.4), individuals older than 60 years (OR: 0.39; CI: 0.09–1.74; p = 0.217), ASA 3 (OR: 0.8; CI: 0.21–3.03; p = 0.746) and tumours located in the inferior 1/3 of the rectum (OR: 0.79; CI: 0.21–2.97; p = 0.73). On the other hand, the odds of non-response were 6 times higher for cT3 and T4 when compared to cT1 or cT2 (OR: 6.0; CI: 0.64–56.06, p = 0.09).

Table 13 Clinical parameters and TRG

Simple Logistic Regression		OR	95% CI	p-value
Continuous Variables				
BMI		1.029	0.2649 - 3.993	0.968
Age		0.392	0.0887 - 1.735	0.217
Categorical Variables				
Gender	Male	0.542	0.1291 - 2.272	0.406
	Female			
Tumour Location	Superior 1/3			
	Medium 1/3	0.791	0.2107 - 2.972	0.732
	Inferior 1/3	0.542	0.1291 - 2.272	0.406
ASA	1+2			
	3	0.800	0.2114 - 3.028	0.746
CRM MR	Free			
	Threatened, invaded	1.169	0.3162 - 4.320	0.817
Extramesorectal nodes	Negative			
	Positive	0.542	0.1291 - 2.272	0.406
cT	T1-2			
	T3-4	6.000	0.6421 - 56.062	0.090
cN	0			
	+	0.350	0.0289 - 4.246	0.399
cM	0			
	1	1.333	0.0770 - 23.085	0.845
Chemotherapy	Capecitabine	0.342	0.0280 - 4.320	0.390
	5-FU			

Simple logistic regression analysis using TRG as dependent variable (TRG 3) and clinical/ molecular variables as independent variables. OR odds ratio of non-response (TRG 3), TRG: tumour regression grade, CI confidence interval, BMI body mass index, ASA American Society of Anaesthesiologists, CRM circumferential resection margin, MR magnetic resonance.

miR-21 Expression and TRG

To study a possible association between miR-21 expression and TRG, we resorted to ROC curve analysis to determine the optimal cut-off that maximized sensitivity, specificity and distinction between responders and non-responders. We found increased odds of non-response in patients with higher miR-21 expression (>1.2) in pre-CRT non-neoplastic rectal tissue (OR: 1.2; CI: 0.24–6.06, p = 0.828) and in patients with levels higher than 2.61 in pre-CRT tumour tissue (OR: 1.6; CI: 0.40–6.63, p = 0.49) (Table 14). Regarding plasmatic miR-21, there was also an increased odds of TRG 3 in patients with pre-CRT miR-21 expression higher than 0.54 (OR: 1.2; CI: 0.24–6.06, p = 0.828) and in patients with post-CRT miR-21 levels >0.84 (OR: 1.09; CI: 0.28–4.33, p = 0.9) (Table 14). Overall, in our sample, patients with higher levels of miR-21 in pre-CRT tissue and plasma had less response to CRT.

Table 14 miR-21 expression and TRG.

Variables		OR	95 % CI	p value
miR-21	≤1.2			
pre-CRT non-neoplastic	>1.2	1.20	0.237 - 6.064	0.8282
miR-21	≤2.61			
pre-CRT tumour	>2.61	1.63	0.402 - 6.625	0.4985
miR-21	≤0.54			
pre-CRT plasma	>0.54	1.20	0.237 - 6.064	0.8282
miR-21	≤0.84			
post-CRT plasma	>0.84	1.09	0.276 - 4.330	0.9

Simple logistic regression according to cut-offs determined with ROC curve analysis.
OR odds ratio of non-response (TRG 3), CI confidence interval.

Clinical Parameters and miR-21 Expression in Pre-CRT Tumour Tissue and Plasma

In pre-CRT tumour tissue an increased odds of miR-21 overexpression (>2.61 fold change) was observed in patients with cT3-4 (OR: 2.71; 95% CI: 0.44–16.68, p = 0.28), TRG 3 (OR: 1.63; 95% CI: 0.40–6.63, p = 0.498), local (OR: 1.14; 95% CI: 0.07–20.02, p = 0.928) and distant recurrence (OR: 2.73; 95% CI: 0.42–17.65, p = 0.289). On the contrary, high miR-21 levels were less likely for subjects older than 60 years (OR: 0.83; 95% CI: 0.19–3.72, p = 0.81), obese (OR: 0.38; 95% CI: 0.08–1.69, p = 0.21) and ASA 3 (OR: 0.41; 95% CI: 0.09–1.81, p = 0.24) (Table 15).

Regarding pre-CRT circulating miR-21, there was an increased probability of miR-21 overexpression (>0.54 fold change) in patients with TRG 3 (OR: 1.79; 95% CI: 0.45–7.19, p = 0.414), N+ (OR: 1.75; 95% CI: 0.14–21.44, p = 0.663) and distant metastasis (OR: 2.21; 95% CI: 0.07–21.22, p = 0.896). However, overexpression was less likely in obese patients (OR: 0.89; 95% CI: 0.22–3.66, p = 0.87), cT3 and cT4 (OR: 0.80; 95% CI: 0.14–4.70, p = 0.80) and in the presence of distant recurrence (OR: 0.30; 95% CI: 0.07–2.45, p = 0.32) (Table 16). Again, overall, patients with miR-21 overexpression in pre-CRT tumour tissue and in blood had less response to CRT.

Table 15 Association between clinical parameters and miR-21 in pre-CRT tumour tissue

Variables		OR	95 % CI	p value
Age	<60			
	≥60	0.83	0.19 - 3.72	0.814
Sex	Male			
	Female	2.1	0.49 - 8.99	0.322
BMI	Low weight + normal			
	Pre-obesity + obesity	0.38	0.08 - 1.69	0.206
ASA score	3			
	2	0.24	0.09 - 1.81	0.409
Stage pre-CRT	I + II			
	III + IV	0.88	0.57 - 27.24	0.203
cT	T1			
	T3+4	2.71	0.44 - 16.68	0.280
cN	0			
	1	0.79	0.05 - 15.33	0.928
pTRG	TRG 0+1+2			
	TRG 3	1.63	0.40 - 6.63	0.498
Distant recurrence	No			
	Yes	2.73	0.42 - 17.65	0.289
Local recurrence	No			
	Yes	1.14	0.07 - 20.02	0.928

Simple logistic regression analysis using miR-21 expression (>2.61-fold change) as dependent variable and clinical variables as independent variables. OR analysis. OR of miR-21 > 2.61 fold change, OR odds ratio, CI confidence interval, ASA American Society of Anaesthesiologists, BMI body mass index, CRT chemoradiotherapy.

Table 16 Association between clinical parameters and miR-21 expression in pre-CRT plasma

Variables		OR	95 % CI	p value
Age	<60			
	≥60	4.14	0.71 - 24.16	0.106
Sex	Male			
	Female	1.73	0.40 - 7.46	0.465
BMI	Low weight + normal			
	Pre-obesity + obesity	0.89	0.22 - 3.66	0.873
ASA score	2			
	3	1.75	0.43 - 7.17	0.442
Stage pre-CRT	I + II			

	III + IV	0.82	0.05 - 14.39	0.896
cT	T1+T2			
	T3+T4	0.80	0.14 - 4.70	0.808
cN	N0			
	N1	1.75	0.14 - 21.44	0.663
cM	M0			
	M1	2.21	0.07 - 21.22	0.896
pTRG	TRG 0+1+2			
	TRG 3	1.79	0.45 - 7.19	0.414
Distant recurrence	No			
	Yes	0.40	0.07 - 2.45	0.320

Simple logistic regression analysis using miR-21 expression (> 0.54-fold change) as dependent variable and clinical variables as independent variables. OR analysis. OR of miR-21 > 0.54 OR odds ratio, CI confidence interval, ASA American Society of Anaesthesiologists, BMI body mass index, CRT chemoradiotherapy, pTRG pathological tumour regression grade.

miR-21 Expression and Oncological Outcomes

With a median follow up of 603 (196–1007) days, we report 3 (8%) mortality cases, 2 (5%) cases of local recurrence (LR) and 7 (19%) of distant recurrence (DR). The low number of death cases precluded correct estimation of overall survival (OS) but 3 and 5-year predicted disease free survival (DFS) were 67 and 46%, respectively (Figure 16).

The overall recurrence HR was increased in women (HR: 1.218, p = 0.797), older patients (HR: 1.64, p = 0.65), lower tumour location (HR: 4.03, p = 0.19), threatened or invaded CRM (HR: 2.14, p = 0.37) and TRG 3 (HR: 3.95, p = 0.11) (Table 17). Overall recurrence HR also augmented in individuals with higher pre-CRT tumour tissue miR-21 expression (HR 2.175, p = 0.37) (Table 17). As expected, there was an impact in 3-year DFS in relation to histological grade (p = 0.09) and distant metastasis (p = 0.029) (Figure 15) but no influence was noted regarding age, gender, T or N stage, tumour location, threatened or invaded CRM, N1c or EMVI.

There was also a decrease in 3-year DFS in patients with higher pre-CRT tumour miR-21 (p = 0.36) and in patients with lower miR-21 in pre-CRT non-neoplastic tissue (p = 0.09) and plasma (p = 0.14). We also evaluated the correlation between pre- and post-CRT circulating and tissue miR-21. Results showed, however, very week correlations (Fig. 16). There was a positive but frail correlation between pre-CRT plasma and tumour miR-21 with an increase in tissue miR-21 with escalation expression in blood (r = 0.002, p = 0.993).

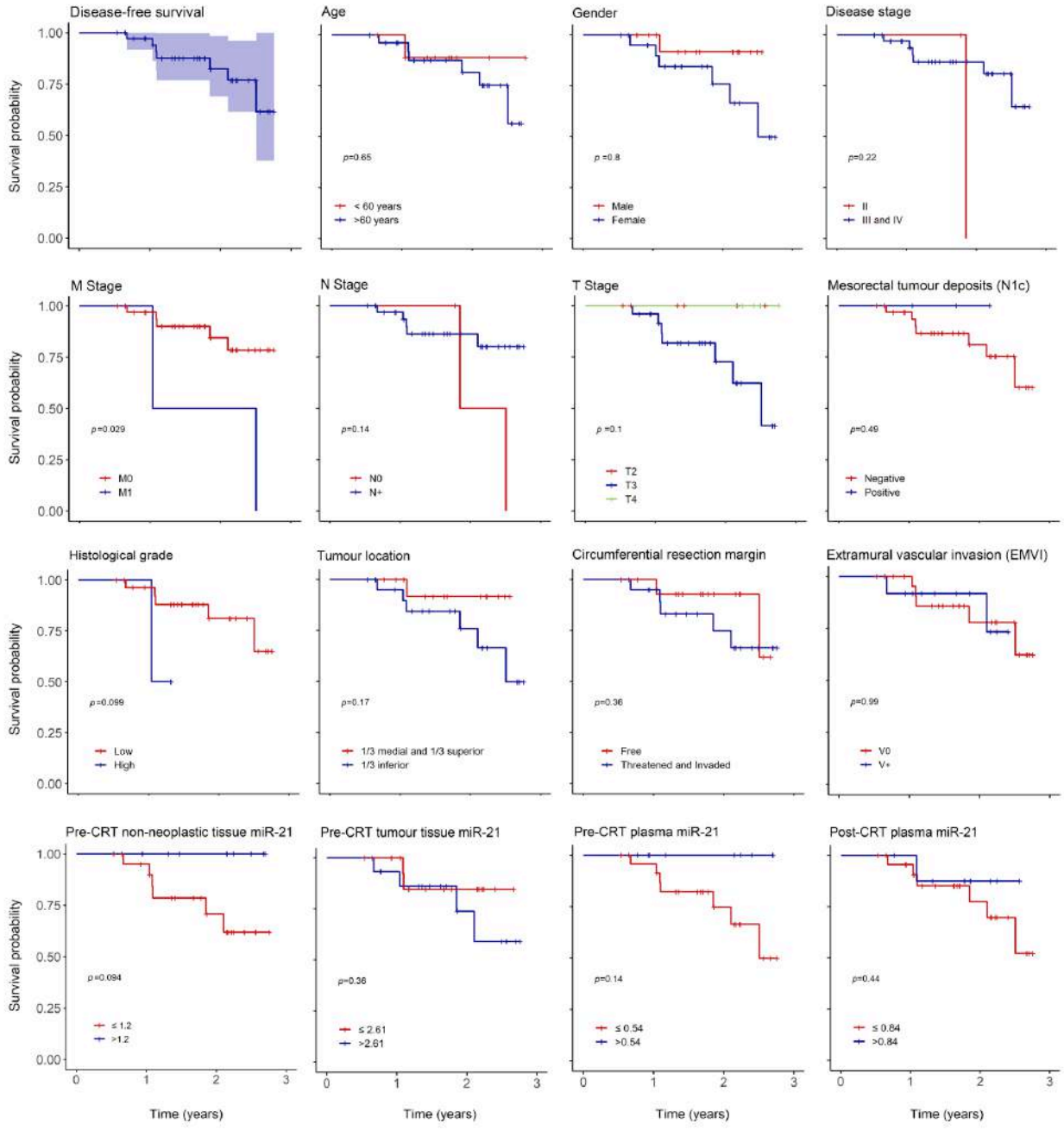

Figure 15 Disease-free survival (DFS) and according to clinical and oncological parameters. Kaplan–Meier curves estimating 3-year overall DFS in patients expressing miR-21 and according to age, gender, disease stage, M stage, N stage, T stage, mesorectal tumour deposits (N1c), histological grade, tumour location, circumferential resection margin (CRM), extramural vascular invasion (EMVI), pre-CRT non-neoplastic tissue miR-21, pre-CRT tumour tissue miR-21, pre-CRT plasma miR-21 and post-CRT plasma miR-21. Reproduced from *Evaluation of tissue and circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer, Pharmaceuticals 2020, 13, 246*

Table 17 Association between clinical parameters, miR-21 levels and overall recurrence

		Total	DFS	r Mean	Simple Cox proportional Hazard model	
					HR	p value
Tumour location	Superior + medium	15	1	2.53	4.027	0.199
	Inferior	22	6	2.25		
Age	<60	10	1	2.54	1.637	0.651
	≥60	27	6	2.38		
Gender	Male	25	4	2.41	1.218	0.797
	Female	12	3	2.39		
CRM	Free	17	2	2.53	2.135	0.368
	Threatened/ invaded	20	5	2.30		
TRG	0-2	21	2	2.57	3.950	0.108
	3	16	5	2.21		
miR-21 pre-CRT tumour	≤ 2.61	17	2	2.47	2.175	0.37
	> 2.61	15	4	2.26		
miR-21 pre-CRT plasma	≤ 0.54	18	5	2.27	0.464	0.36
	>0.54	15	2	2.45		

Simple Cox Proportional Hazards Model using global recurrence as dependent variable and clinical parameters as independent variables. HR: hazard ratio; CRM: circumferential resection margin; TRG: tumour regression grade; DFS: disease free survival.

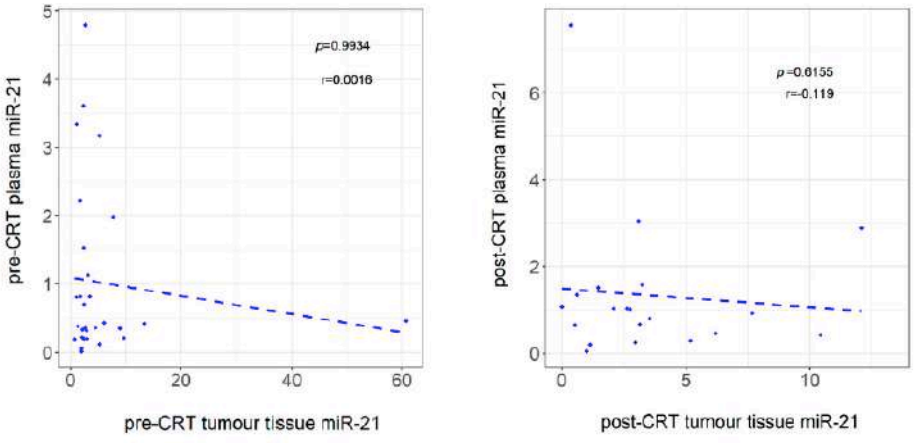

Fig 16 Correlation between pre- and post-CRT miR-21 expression in plasma and tumour tissue. Reproduced from *Evaluation of tissue and circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer, Pharmaceuticals 2020, 13, 246*

DISCUSSION

The interest in identifying biomarkers for cancer has led both researchers and clinicians to focus on miRNAs [269]. Some studies have investigated the diagnostic and prognostic value of miR-21 in RC as well as its potential to predict response to CRT [257],[266],[267]. However, the conclusions obtained from these studies were inconsistent granting the need to further explore the clinical significance of miR-21 as a biomarker in this setting.

Generally, findings associate a superior miR-21 expression with a non-or incomplete response. In fact, in the previous retrospective study, our group identified an association between miR-21 overexpression in pre-CRT rectal tumour tissue and worse pathological response. In that study, this miRNA could differentiate incomplete from complete responders and potentially be used as biomarker to predict TRG. Nevertheless, the evaluation of circulating miR-21 as a non-invasive biomarker of response to CRT in rectal cancer has never been investigated.

The first detection of miRNAs in body fluids occurred when miR-21 was found in the serum of B-cell lymphoma patients [270]. Since then, up-regulated miR-21 levels in plasma have been associated with solid cancers (glioblastoma, breast cancer, and pancreatic cancer) [271] and therefore it was termed oncomiR.

Levels of miRNAs in plasma are remarkably stable, reproducible, consistent among individuals of the same species [272] and cells actively release the majority of circulating miRNAs. The idea of a correlation between circulating and tissue miRNA supports the hypothesis that plasmatic miRNAs can serve as biomarkers of disease or disease response. miRNAs appear to demonstrate the same change in expression, either increased or decreased, in plasma or serum and tumour tissues of patients with various types of cancer [272]. However, only few studies focused on circulating miRNAs in CRC patients [274]-[276].

Clinical significance of circulating miR-21 levels in CRC remains, in fact, not fully understood. Some studies report on seric miR-21 as a discriminative biomarker of colorectal neoplasms from healthy controls [196],[202],[203],[252],[276]-[277] and from benign or premalignant adenoma [278]. Circulating miR-21 has also been correlated with tumour size, grade of differentiation, invasion, metastasis, recurrence, and survival [201],[252]. The expression of miR-21 has been found significantly increased in preoperative serum from CRC patients and this correlated with tumour size, poor survival, and LN metastasis [239],[277].

Another important issue is that, in reality, very few studies differentiate between colon and rectal cancer patients and these are two different entities with distinct treatment options. In fact, serum miR-21 levels seem to be upregulated in RC tissue in comparison to colon cancer [279].

In the present work, we aimed to investigate the potential of tissue miR-21 as a biomarker of response to CRT in a prospective group of RC patients and validate our previous retrospective results [280]. Likewise, we also intended to assess circulating miR-21 in this setting.

Although we could not demonstrate the efficacy of tissue and plasma miR-21 to differentiate responders from non-responders, we did find an odds increase of non-response in all patients expressing higher miR-21 levels. miR-21 was upregulated in tumour tissue and there was an increased probability of pre-CRT tumour tissue miR-21 overexpression in patients with non-response. In addition, in this study overall recurrence HR increased in patients with less response, threatened or invaded CRM, and higher pre-CRT tumour tissue miR-21 levels. Regarding 3-year DFS analysis, we observed a decrease in survival in patients with higher miR-21 levels in pre-CRT tumour tissue, while overexpression of miR-21 in pre-CRT non-neoplastic tissue was related to a better survival. This is concordant with our hypothesis that when comparing pre-CRT non-neoplastic and tumour tissue we predict response to treatment, where higher miR-21 in pre-CRT tumour tissue in comparison with non-neoplastic tissue is indicative of a worse response to treatment, whereas equal or higher miR-21 in pre-CRT non-neoplastic tissue is associated with better response to CRT. Considering plasma miR-21 analysis, although with no statistical significance, we observed increased odds of pre-CRT circulating miR-21 overexpression in non-responders.

Overall, these results are in line with our retrospective study that found a significant association of miR-21 overexpression in pre-CRT RC tissue with worse response to neoadjuvant therapy. Moreover, pre-CRT plasmatic miR-21 may be also related to less response. To our knowledge, this is the first report in which circulating miR-21 has been investigated as a predictive biomarker of response to neoadjuvant CRT in RC.

Recently, it was observed that circulating exosomal miR-21 could distinguish chemotherapy resistant from chemosensitive CRC patients [281]. This miRNA was shown to be upregulated in the exosomes of chemoresistant CRC cell lines and in pre-chemotherapy exosomal serum of patients that did not respond to treatment. These results are in accordance to our suggestion that overexpression of pre-CRT circulating miR-21 may be indicative of worse response to CRT in RC setting, possibly related to the ChT effect.

CONCLUSION OF BOTH STUDIES

The results of these 2 studies are concordant and show that miR-21 overexpression in pre-CRT RC tissue or plasma have worse response to neoadjuvant therapy, demonstrating the potential of this miRNA as a biomarker of response to CRT.

The differences observed between studies are probably related to the limitation in sample size in the prospective study as well as the different TRG based definition of patient groups. Besides, although both groups of patients include uniform sampling and treatment, there is a potential for intratumoral heterogeneity.

As one of the most well-established oncogenic miRNAs in RC, miR-21 plays a key role in biological processes needed for tumorigenesis, including resistance to apoptosis and replicative immortality. An association has been found between radioresistance and the expression of p53, RAS, raf-1, bcl-2 and cell stemness. In fact, miR-21 exerts its oncogenic function mainly through the suppression of genes participating in apoptosis particularly bcl-2, and is a negative regulator of p53 signalling. Also, targeting miR-21 reduces the number of CSCs during 5-FU treatment. Finally, miRNAs 21 has been described to induce resistance to 5-FU, a possible mechanism of CRT resistance. In summary, miRNA-21 seems to have a major interference with response to chemoradiotherapy in RC.

The results presented here provide an association between miRNA-21 in RC neoadjuvant therapy setting and tumour regression with significant implications that strengthen its the role as predictor of response. The definitive impact as a predictive tool for pathological response in patients treated with CRT needs to be established in larger cohorts.

Of note, in addition to possible biomarkers, miRNAs may be potential therapeutic targets via re-introducing miRNAs absent in carcinogenic pathways or by inhibiting oncomiRs [193][241][282]. Affecting miRNAs implicated in the mechanism of resistance to CRT may improve the therapeutic outcome.

The biggest challenge will continue to be the identification of miRNA targets that shed light on our understanding of downstream cellular mechanisms of resistance to CRT.

Ongoing research: The acknowledgment of the importance of identifying factors predictive of response to medical therapies and of understanding the mechanism of tumorigenesis led to further exploratory research on the role of metabolism-related hormones in RC. This translated in a study, currently ongoing, evaluating the influence of these hormones on response to CRT and their correlation with CSC markers. Also, to further study oncogenic mechanisms and test new anticancer drugs/ regimens we started to implement a 3D model of patient-derived RC organoids (Chapter 6).

Chapter 4

Surgical Determinants in the Treatment of Rectal Cancer

Transanal Total Mesorectal Excision

Local Excision in Post Chemoradiotherapy Patients

Factors Predictive of Complications Related to Loop Ileostomy

INTRODUCTION

The impact of the surgeon and surgical technique in RC patients' outcomes is undisputable. We evolved from performing one unique technique in all patients, independently of the tumour characteristics, to a multiple of therapeutic options, selected to obtain the best possible outcomes. We translated from a totally non-selective approach to choosing surgical option according to patient performance status, tumour parameters, oncological risk or even response to neoadjuvant therapies. If, initially, no relevance was given to the morphology of the TME specimen, the acknowledgment of an association between the plane of surgery, mesorectal integrity and locoregional recurrence emphasized the need for a good quality surgery. Likewise, if originally the main goal was patient's survival, we progressed to endorsing not just oncological outcomes but also functional ones, with organ-preserving options increasingly claimed for.

Indeed, RC surgery has become very demanding and choosing the right procedure for the right patient is a great responsibility. But if we consider that there are biological determinants that influence results, we must concede the importance of the surgical options and surgeon's ability on the outcomes of patients.

In this complex setting, as new techniques develop and old dogmas are questioned, it is imperative that rectal surgeons audit their results, compare techniques and search for surgical determinants that impact on outcomes. Our goal should be to obtain the best possible results minimizing treatment morbidity.

With growing knowledge on RC, many unanswered questions remain. This Chapter approaches some of the controversial issues currently being debated in RC, specifically the novel surgical option of transanal TME (TaTME), the morbidity of the loop ileostomy and the safety of non-radical strategies after neoadjuvant therapy.

Can we improve results by introducing a new technique in RC treatment, namely TaTME? Is this technique comparable to the standard-of-care laparoscopic TME regarding short and long-term outcomes?

Is it safe to admit a more conservative local excision approach for patients post CRT?

Can we identify factors predictive of complications related to stoma and better select patients for derivative ileostomy?

These are the questions on the basis of this Chapter.

Transanal total mesorectal excision (TaTME)

Rectal cancer (RC) gold standard surgical treatment is TME. The application of laparoscopy to the treatment of this disease (laparoscopic total mesorectal excision - lapTME) has been a major technical leap that brought advantages in short and long-term outcomes. LapTME can, however, be very challenging, specially in obese male patients with distal tumours due to the difficulties of pelvic dissection related to limited operative field, decreased mobilization and stapling [35],[283],[284].

In previous randomized controlled trials (RCT) lapTME has been associated with high conversion (up to 34%), anastomotic leak (up to 19%), incomplete mesorectum, invasion of CRM and of distal margin (up to 18%) [8],[113] with acknowledged impact on oncological outcomes [6],[7]. Also, it is associated with sexual dysfunction (up to 38%), urinary dysfunction (up to 26%) and major low anterior resection syndrome (LARS) (58% at 6 months; 49% at 12 months) [8],[113].

As circumferential, distal margins and mesorectal integrity are the most important prognosis factors for LR, the relevance of a good quality surgery is emphasized [6]. Risk factors for positive CRM and intraoperative technical difficulty are precisely male gender, high BMI, narrow pelvis, distally located and advanced T-stage lesions [35],[283],[284]. In fact, lower cancers have worst oncological outcomes and higher LR. If on one hand we must guarantee optimal surgical outcomes, on the other TME can be technically challenging due to the difficulty of working in a restrict space with limited vision. In this setting, TaTME is the most recent surgical method developed to overcome technical difficulties in pelvic approach.

This reverse proctectomy (down-to-up) performed with laparoscopic instruments through the anal canal was developed by Patricia Sylla and Antonio Lacy in 2010 [10]. It is indicated for the treatment of mid and low rectal cancer but also to inflammatory bowel disease (IBD), complex fistulae or revision of colorectal anastomosis.

Performed in a two teams approach working synchronously, TaTME has several potential advantages namely a better view of the prostate and recto-vaginal septum with superior dissection of anterior tumours and ability to decide whether to stay in front or behind Denonvilllier's fascia, better visualization of the pelvic floor muscles and nerve bundles, reduced specimen manipulation due to the pneumorectum that aids is dissection, determination of an appropriate distal margin and better washout [286]-[288] Potential gains from this technique are an easier dissection in the male narrow pelvis, a decrease in conversion, an increase in sphincter saving resections, better anastomotic techniques with subsequent lower morbidity, improved specimen quality and a decrease in surgical site infection [288],[289]. TaTME dos not imply stapling of the rectum distally to the tumour, which avoids imperfect firing (due to the limitation of the angulation to 45º), "dog ears" and crossing of staple lines. In classic laparoscopy, low pelvic tumours in male obese patients oblige several stapling, with known impact on anastomotic leak [10],[290],[291].

However, TaTME has specific challenges associated with the change in anatomic perspective and the demands of single-port technique. Likewise, it also introduced new complications, not commonly associated with the classic and laparoscopic approaches, such as urethral injuries and carbon dioxide embolism [115],[292],[293].

Several authors report TaTME short-term results as similar or better than standard laparoscopic resection regarding conversion, anastomotic leak, involvement of distal and circumferential margins, mesorectal integrity, LN yield, operative time, blood lost, morbidity, length of hospital stay and readmission rates [290],[294]-[308]. Outcomes regarding function are sill controversial, albeit most studies presenting comparable results [306]-[310]. Although short-term clinical outcomes seem to be well established, there are still inconsistencies regarding oncological and also long-term ones [310],[311].

TaTME was started in the Rectal Cancer Group of Hospital Beatriz Ângelo in March 2016. Prior to introducing this technique, institutional protocols and procedural guidelines that integrated the possibility of patients with mid and low RC being treated with this technique were developed by the PhD candidate.

Also, before endorsing TaTME technique, this unit's colorectal surgeons underwent hands-on courses and observation of live procedures with Professor Antonio Lacy at Hospital Clinic in Barcelona and Professor Joep Knol at Jessa Hospital in Hasselt, Belgium. Didactic learning through iLapp platform was also engaged and the first cases done in Portugal by the surgeons were mentored by Professor Joep Knol and Professor Roel Hompes. Moreover, these proctors also came to our institution for a TaTME Masterclass with live cases performed with the surgeons of our Unit.

Moreover, in the context of the worldwide introduction of a new technique and acknowledging the importance of contributing to international databases and multicentric studies, the PhD Candidate initiated a cooperation with the International TaTME Registry. The collaboration meant the scrutiny and inclusion in the Registry of data regarding all patients submitted to this technique. This contribution led to the publications of 3 articles, with the Candidate as collaborator (Chapter 8):

- Incidence and Risk Factors for Anastomotic Failure in 1594 Patients Treated by Transanal Total Mesorectal Excision: Results From the International TaTME Registry. M Penna , R Hompes, S Arnold *et al*, International TaTME Registry Collaborative Ann Surg 2019 Apr; 269(4):700-711[312];

- Predictive Factors and Risk Model for Positive Circumferential Resection Margin Rate After Transanal Total Mesorectal Excision in 2653 Patients With Rectal Cancer. S Roodbeen, FB de Lacy, S van Deren *et al*. International TaTME Registry Collaborative Ann Surg 2019 Nov; 270(5):884-891[284]

- Carbon Dioxide Embolism Associated With Transanal Total Mesorectal Excision Surgery: A Report From the International Registries. EA Dickson, M Penna, C Cunningham *et al*. International TaTME Registry Collaborative. Dis Colon Rectum. 2019 Jul;62(7):794-801[292]

Original Paper 4

Transanal Total Mesorectal Excision: 3-year Oncological Outcomes

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ABSTRACT

Background: *Rectal cancer treatment has evolved with the implementation of new surgical techniques. Transanal total mesorectal excision is the most recent approach developed to facilitate pelvic dissection of mid- and distal rectal tumours.*

Objectives*; The purpose of this study was to analyse the short- and mid-term oncological outcomes of TaTME.*

Methods: *A study was conducted on patients treated with transanal total mesorectal excision for rectal cancer at two colorectal units in Portugal between March 2016 and December 2018. Clinical, pathological and oncological data were retrospectively analysed. Primary endpoints were 3-year overall survival, disease-free survival and local recurrence. Secondary endpoints were clinical and pathological outcomes.*

Results*: Fifty patients (31 males, [62%], median age 66 years [range 40–85 years]) underwent transanal total mesorectal excision, 49 (98%) for malignant and 1 (2%) for pre-malignant disease. There were no cases of conversion, 49 (98%) patients had complete or near-complete mesorectum, all resections were R0 with adequate distal and circumferential margins. With a median follow-up of 36 months, there were 2 cases (4%) of local recurrence and 3-year estimated overall survival and disease-free survival were 90% and 79%, respectively.*

Conclusions: *TaTME can provide safe mid-term oncological outcomes, similar to what has been published for classic and laparoscopic TME. Our results also show how demanding this novel approach can be and the consequent need for audited data and standardized implementation.*

Keywords: rectal cancer; TaTME; oncological outcomes

RATIONAL AND AIMS

Rational: TaTME is a technique recently introduced for the treatment of rectal cancer. In this setting, it is most relevant that each group audit their results for a safe implementation of any technique.

Aim: This study's objective was to investigate short and mid-term clinical and oncological outcomes of the introduction of TaTME in a colorectal Group and to show the outcomes of the first 50 cases, corresponding to the learning curve of the technique, accepted as 20-25 cases per surgeon [314]-[316].

MATERIALS AND METHODS

This was a retrospective study of prospectively analysed data. The first 50 consecutive patients with rectal cancer stage I-IV, AJCC submitted to TaTME between March 2016 and December 2018 in Hospital Beatriz Angelo and Hospital da Luz, Lisbon were eligible for this study. The unit's volume of rectal radical resection during the elected study period is shown (Fig. 1). Initially, all patients with RC (less than 15 cm from the anal verge) were considered elective for TaTME but, due to the possibility of performing unneeded too distal anastomosis, we changed the selection to patients with cancers of the mid and lower rectum (from 10 to 5 cm and less than 5 cm from the anal verge, respectively, through rigid sigmoidoscopy and magnetic resonance). All patients accepted this technique through informed consent. Data was gathered from the electronic hospital databases.

Pathological specimen plane was defined according to Quirke *et al* as '*muscularis propria* plane', mesorectal plane' or the 'intramesorectal' [7]. In this study, anastomotic leak was defined according to the International Study Group of Rectal Cancer, including subclinical radiological and clinical leak, pelvic and perianastomotic abscess [316]. Post-operative morbidity was assessed according to Clavien-Dindo Classification [317] and included all complications related to the initial surgery, even after 30 days. Data was obtained from the hospital's electronic database. Primary endpoints were 3-year overall survival (OS), disease- free survival (DFS) and LR. Secondary endpoints were clinical and pathological outcomes

Statistical analysis

This was a descriptive study. Survival analysis was performed through Kaplan Meier statistics. Overall survival (OS) was calculated considering surgery date until death date. Disease-free survival (DFS) was estimated considering surgery date until the appearance of recurrence, local or distant. SPSS (IBM, version 20) and R (version 3.0.2) were used. $P \leq 0.05$ was considered to be statistically significant. There were no missing data and no patients were lost to follow-up. Continuous variables were reported as n, median and range of lower and higher values.

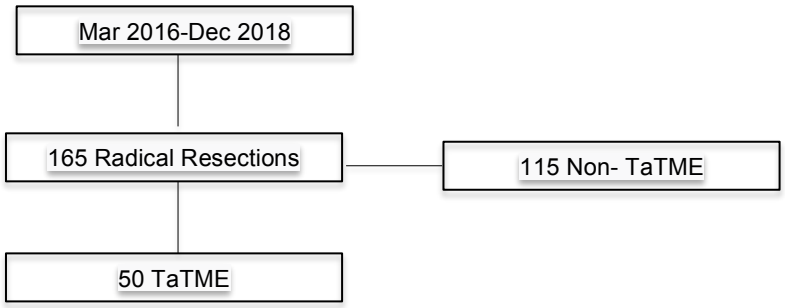

Figure 1 Volume of radical resections for rectal cancer. Unit's volume of radical resections (non exenterative) for rectal cancer during the elected period of the study.

RESULTS

Patient Clinical parameters

During the study period, a total of 50 patients underwent TaTME, (31 [62%] males, median age 66 years (range 40–85 years) with a median BMI 26 kg/m2 (range 19–39 kg/m2). Forty-eight (96%) patients had an Eastern Cooperative Oncology Group (ECOG) performance status score of 0, 35 (70%) patients were classified as American Society of Anaesthesiologists (ASA) class II and 7 (14%) had the previous laparotomy for other causes (Table 1).

Table 1 Patient clinical parameters

Clinical parameters		Patients (n = 50)
Gender, n (%)	Male	31 (62)
	Female	19 (38)
Age, median		66 (40-85)
BMI, median		26 (19-39)
PS (ECOG), n (%)	0	48 (96)
	1; 2	2 (4)
ASA score, n (%)	II	35 (70)
	III	15 (30)
Previous abdominal surgery, n (%)	Histerectomy	2 (4)
	Colectomy	2 (4)
	Appendectomy	2 (4)
	Anterior resection	1 (2)

BMI Body Mass Index, PS performance status, ECOG Eastern Cooperative Oncology Group, ASA American Society of Anaesthesiologists. Continuous variables are reported as median, range of lowest and higher value

Preoperative staging and neoadjuvant therapy

Of the 50 patients in the study, 49 (98%) were treated for RC and 1 (2%) for endoscopically non-resectable tubulovilous adenoma with high-grade dysplasia. The neoplasia was localized mainly in the mid and low rectum with a median distance to the anal verge of 70 mm (range 20–120 mm). All patients underwent preoperative staging with pelvic MR and CT scan of the chest and abdomen except for 2 that underwent ERUS due to metallic prostheses. Pelvic MR showed mesorectal fascia invaded or threatened in 10 (20%) patients and EMVI in 5 (10%). The median level of CEA was 1.35 ng/mL (range 1.3–1.4 ng/mL). Twenty-four (48%) patients had neoadjuvant CRT, 23 with a long-course (LCCRT) and 1 with a short-course (SCRT) regimen. Restaging pelvic MR was done at 6 weeks post neoadjuvant CRT and 11 (46%) patients showed a good response, tumour regression grade 1 or 2 [316] (Table 2).

Table 2 Pre-operative staging and neoadjuvant therapy

Pre-operative staging and neoadjuvant therapy		Patients (n = 50)
Disease	Malignant	49 (98)
	Benign	1 (2)
Location, rectum (%)	1/3 superior	3 (6)
	1/3 medium	30 (60)
	1/3 inferior	17 (34)
Tumour extension (mm), median		58 (5 - 120)
Distance to anal verge (mm), median		20-120 (70)
cT	> T3	25 (50)
cN	Positive	25 (50)
CRM, n (%)	Free	39 (78)
	Threatened	3 (6)
	Invaded	7 (14)
	NA	1 (2)
EMVI, n (%)	Negative	44 (88)
	Positive	5 (10)
	NA	1 (2)
CEA (mg/mL)		1.35 (0.5 - 296)
CRT	No	26 (52)
	LCCRT	23 (46)
	SCRT	1 (2)
mrTRG, n (%)	mrTRG 1 e 2	11 (46)
	mrTRG 3	3 (12)
	ND	10 (42)
	NA	26

cT and cN - American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017, CEA carcinoembrinonary antigen, CRM magnetic resonance accessed circumferential resection margin; EMVI magnetic resonance accessed extramural venous invasion, CRT chemoradiotherapy, LCCRT long course chemoradioth erapy, SCRT short course chemoradiotherapy, mrTRG magnetic resonance accessed post CRT Tumour Regression Grade [7], NA not applicable, ND not discriminated. Continuous variables are reported as median, range of lowest and higher values.

Surgical technique

All 50 patients had preoperative mechanical bowel preparation and underwent the surgical procedure at a median of 12 weeks (range 7–22 weeks) after CRT. Procedures were done with a synchronous 2-team approach, transabdominal and transanal, by the same surgeons. There was no intraoperative mortality (Table 3).

Abdominal approach

The abdominal approach was performed through laparoscopy in 42 (84%) patients and robotically assisted in 4 (8%) patients, for a total of 46 (92%) treated with a minimally invasive approach (Table 3). There were 4 (8%) abdominal conversions to midline laparotomy, 1 due to intolerance of pneumoperitoneum, 1 due to pre-sacral bleeding and 2 due to technical difficulty related to obesity.

Complete mobilization of the splenic flexure was done in all cases and proximal inferior mesenteric pedicle ligation was performed in 40 (80%) cases. Concomitantly with rectal resection, 2 resections of liver metastasis, 1 total colectomy and 2 protocolectomies were performed, all laparoscopically. Forty-seven anastomoses were fashioned, 40 (85%) mechanical and 7 (15%) handmade, predominantly side- to- end (72%), with a median distance to the dentate line of 20 mm (range 0–70 mm). All patients with a primary anastomosis had a protective loop ileostomy.

The surgical specimen was extracted through a Pfannenstiel incision in 39 (85%) cases and pelvic drainage was placed in 24 (48%) patients. Median intraoperative blood loss was 100 mL (range 50–2000 mL), with only 1 patient requiring transfusion due to pre-sacral bleeding (Table 3). Median operative time was 285 min (range 202–445 min). Regarding the evolution of the learning curve, there was no difference in operative time in the first (median: 295 min; range 212–430 min) and last 25 patients (median 285 min; range 202–445 min).

Transanal approach

For the transanal approach, *Lone Star® Retractor* (*Cooper Surgical*, USA) and *GelPOINT®Path Transanal Access Platform* (*Applied Medical*, USA) were used. No conversions occurred. There were 2 (4%) intraoperative complications, 1 urethral and 1 vaginal lesion, both immediately repaired (Table 3).

Table 3 Surgical technique

Surgical Technique		Patients (n = 50)
CRT- surgery, (weeks), median		12 (7-22)
Abdominal approach, n (%)	Laparoscopy	42 (84)
	Laparotomy	4 (8)
	Robotic	4 (8)
Conversion, n (%)	Abdominal	4 (8)
	Transanal	0 (0)
Anastomosis, n (%)	Mechanical	40 (85)
	Hand-sewn	7 (15)
Anastomosis, n (%)	Side-to-end	34 (72)
	End-to-end	11 (24)
	Ileoanal pouch -anal	2 (4)
Anastomosis distance from dentate line, (mm) median		20 (0-70)
Specimen extraction site, n (%)	Pfannenstiel	39 (85)
	LIF	5 (11)
	Transanal	2 (4)
	NA	4
Operative morbidity, n (%)		
Abdominal approach	Pre sacral bleeding	1 (2)
Transanal approach	Vaginal lesion	1 (2)
	Urethral lesion	1 (2)
Stoma, n (%)	Loop ileostomy	47 (94)
	End colostomy	2 (4)
	End ileostomy	1 (2)
Drains, n (%)		24 (48)
Blood lost (cc), median		100 (50-2000)
Operative time (min), median		285 (202-445)

CRT chemoradiotherapy, LIF left iliac fossa, min minutes, NA not applicable. Continuous variables are reported as median, range of lowest and higher values.

Postoperative period and follow-up

There was a median length of stay of 7 days (range 3–42 days) with a readmission rate of 12% (6 patients). There was no postoperative mortality and 11 (22%) patients had Clavien-Dindo IIIB morbidity [316]. There was no difference in the overall complication rate between the initial and late phase of the learning curve, with 5 versus 6 patients having Clavien-Dindo IIIB morbidity, respectively.

In this study, the anastomotic leak was defined as including subclinical radiological and clinical leak, pelvic and perianastomotic abscess [317]. There were 8 (17%) anastomotic leaks. Of these, 5 had to

be treated with reoperation, 3 through transanal drainage, 1 with transabdominal drainage and only 1 with an end colostomy. 50% of patients that had anastomotic leaks had undergone neoadjuvant CRT. 46 (98%) patients maintained their anastomosis. Until the final date of this study, 44 (94%) had their ileostomies closed with a median time to closure of 29 weeks (range 2–67 weeks) (Table 4).

Table 4 Post-operative period and Follow-up

Post-operative period	
Admission (days), median	7 (3-42)
30-day mortality, n (%)	0 (0)
Readmission, n (%)	6 (12)
Postoperative complications (treatment), n (%)	22 (44)
Clavien-Dindo I	
Ileus	1 (2)
Clavien-Dindo II	9 (18)
Respiratory infection (AB)	2
Bacteriemia (AB)	1
Urinary tract infection (AB)	3
High output ileostomy (loperamide, omeprazol, codein)	1
Anastomotic leak, recto-vaginal fistulae (AB)	1
Anastomotic leak, pelvic abscess (AB)	1
Clavien-Dindo IIIA	
Anastomotic leak, pre-sacral abscess (AB, endosponge)	1 (2)
Clavien-Dindo IIIB	11 (22)
Abdominal wall dehiscence (closure)	1
Pancreatic fistulae (drainage)	1
Intrabdominal haematoma (drainage)	2
Parastomal hernia (suture)	1
Jejunal fistulae (segmental resection)	1
Anastomotic leak	5
Transanal drainage	3
Transabdominal drainage	1
End colostomy	1
Ileostomy closure, n (%)	44 (94)
Time to stoma closure (weeks), median	29 (2-67)
Adjuvant CT, n (%)	23 (46)

Complications according to Clavien-Dindo [7], AB antibiotic treatment, CT chemotherapy. Continuous variables are reported as median, range of lowest and higher values.

Pathological outcomes

Pathology reported 100% of R0 resection, free distal and circumferential margins, with a median node sampling of 19 nodes (range 4–52 nodes) and 49 (98%) good quality specimens graded as in mesorectal or intramesorectal plane [7] (Table 5).

Table 5 Pathological Outcomes

Pathological Outcomes	
Stage, n (%)	
Benign	1 (2)
T0N0M0	4 (8)
I	23 (46)
II	5 (10)
III	16 (32)
IV	1 (2)
Radicality, n (%)	
R0	50 (100)
Specimen quality, n (%)	
Mesorectal plane	40 (80)
Intramesorectal plane	9 (18)
Muscularis propria plane	1 (2)
Nodes, median	19 (4-52)
Free Margin, n (%)	
Distal	50 (100)
Circumferential	50 (100)
Tumour diameter (mm), median	28 (15-45)

Pathological Staging according to the American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017; Specimen quality according to P. Quirke [7]. Continuous variables are reported as median, range of lowest and higher values.

Oncological outcomes

No patients were lost to follow-up and the median follow up time was 36 months (range 14–53 months). There were 2 (4%) cases of LR, one at 8 months, synchronous with distant metastasis, and another at 22 months in a patient with previous distant recurrence. Recurrences were pre-sacral and anastomotic, respectively, with no pelvic lateral sidewall or multifocal pattern. The patient with a pre-sacral recurrence had a suboptimal specimen with an incomplete mesorectum in the TaTME specimen.

LR happened at 8 and 22 months and neither patient had metastasis at initial diagnosis. There were 10 (20%) cases of metachronous distant disease after a median of 8 months (range 1–17 months). Patients who developed distant metastasis were initially in stage IV in 2 cases and stage III in 7. Patterns of distant recurrence related to metastasis in the lung, liver, central nervous system, bone and periaortic nodes (Table 6). Overall, there were 4 deaths, all related to disease progression. One- and 3-year OS were 100% and 90%, respectively (Fig. 2a). 1- and 3-year DFS were 84% and 79%, respectively (Fig. 2b).

Table 6 Oncological Outcomes

Oncological Outcomes	
Follow up time (months), median	36 (14-53)
Time for local recurrence (months), median	15 (8-22)
Time for distant recurrence (months), median	8 (1-17)
Local recurrence	2 (4)
Anastomotic	1 (2)
Presacral	1 (2)
Distant recurrence	10 (20)
Lung	4 (40)
Liver	2 (20)
CNS + bone	2 (20)
Periaortic nodes	2 (20)
Global Mortality	4 (8)

CNS central nervous system

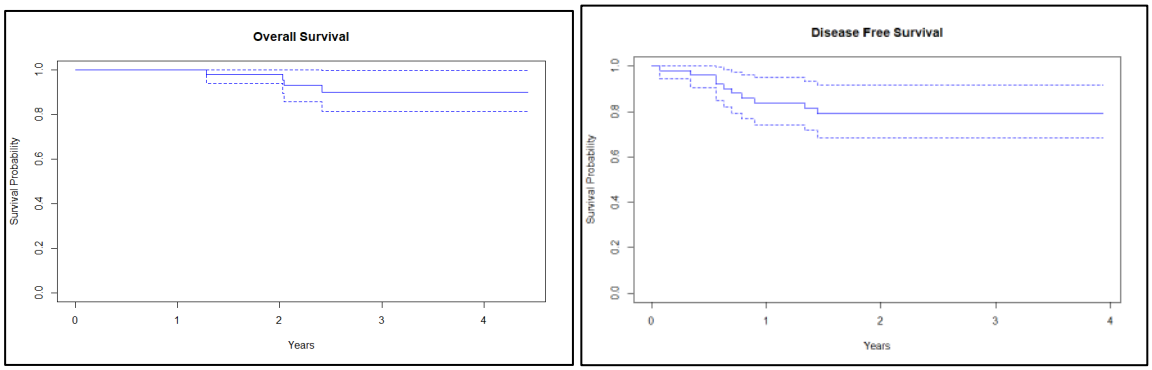

Figure 2 Oncological Outcomes. Kaplan-Meier curves for A) overall survival, B) disease free survival. (A) One and 3-year overall survival were 100% and 90% (B) One and 3-year disease free survival were 84% and 79%.

DISCUSSION

When introducing new techniques in Surgery there must be a scrutiny of outcomes for a safe implementation and it becomes imperative that surgeons report their results. The aim of the present study was to present the mid-term clinical and oncological outcomes of the first 50 TaTME cases of our colorectal team, reflecting the learning curve.

Regarding clinical results, there were no cases of transanal conversion confirming the feasibility of TaTME. There was, however, an intraoperative urethral lesion. This iatrogenic lesion, like pelvic sidewall vascular injury and carbon dioxide embolism, has been associated with the technique [115],[293]. Overall, authors report TaTME short-term results similar to or better than standard laparoscopic resection regarding conversion, anastomotic leak, involvement of distal and circumferential margins, mesorectal integrity, LN yield, operative time, blood loss, morbidity, length of hospital stay, readmission rates and function [294], [301]-[303],[322]. In our work, anastomotic leak was defined as including clinical, radiological leak and perianastomotic/ pelvic abscess [316]. We present an overall early and late anastomotic leak rate of 17% that, despite being high, is concordant with what has been previously published [319]. Nonetheless, in this 8 patients group, only one had their anastomosis taken down with definitive colostomy and all the rest had their loop protective ileostomies reversed.

Overall, intraoperative complications occurred in the initial stage of the learning curve (first 25 patients) with no differences in the evolution of the learning curve related to operative time and postoperative complications. Regarding pathological outcomes, specimen quality was good with 49 (98%) graded as in mesorectal or intra mesorectal plane and 100% with R0, clear distal and circumferential margins.

Although it seems well established that short-term clinical outcomes are good there are still inconsistencies regarding oncological ones. Several authors have reported good oncological outcomes but mostly with a short follow-up time [296],[305],[324]-[328]. Likewise, studies that compare survival between lapTME and TaTME also present good oncological results but, again, with short-term follow-up [297],[301],[308],[322],[329],[330]. In fact, very few studies report on more than 2-year oncological outcomes. A recent multicentre study on 211 TaTME patients demonstrated 3-year OS of 93%, DFS of 80% and 6% of LR [327]. Perdawood *et al* [328] published on 200 TaTME patients and, with a follow-up of 2 years, found 90% OS, 81% DFS, 5% of LR and 12% of distant metastasis. Marks *et a*l. studied 373 patients that underwent trans abdominal trans anal approach (TATA) with the abdominal dissection performed through laparoscopic, pure transanal, open or robotic approach. With a mean follow-up of 66 months (range 0–300 months), 5-year OS was 90% and LR was 7.4% [329],[310]. Recently, Hol *et al* [311] reported that 159 TaTME patients at 3 and 5 years had 84% and 77% OS, 92% and 81% DFS and 2% and 4% LR, respectively. Finally, in a controlled trial with 100 patients randomized to lapTME and Ta

TME, Denost *et al* [313] reported no significant difference between groups regarding 5-year LR or DFS. Until now, the fact that most studies only express short-term oncological outcomes has not allowed definitive conclusions do be drawn. In addition, recent literature has raised concerns about the oncological safety of TaTME with publications reporting early multifocal pelvic cavity and sidewall recurrence [314],[315].

In this setting, the present study had the objective of investigating 3-year outcomes of our first 50 TaTME patients. With a median follow-up of 36 months (range 14-48 months), we report 2 (4%) cases of LR, occurring at 8 and 22 months, none multifocal or related to the pelvic sidewall. The first of these cases related to a patient with an intraoperative urethral lesion and a suboptimal specimen who developed a pre-sacral recurrence at 8 months, which emphasizes the importance of specimen quality and surgical technique. In our cohort, distant metastases were found in 10 (20%) patients after a median of 8 months (range 1–17 months), 2 of who had stage IV disease at initial diagnosis and the other 8 stage III. Three-year OS and DFS were 90% and 79%, respectively.

Although limited by the small number of patients, we intended to show the outcomes of the learning curve of TaTME, accepted to be at least 20-25 cases per surgeon [313],[315],[330]. Our short-term results concerning the oncological safety of TaTME parallel the outcomes published so far.

Original Paper 5

Transanal versus Laparoscopic Total Mesorectal excision. Comparative Study of Long-tem Oncological Outcomes

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ABSTRACT

Background: *Transanal total mesorectal excision (TaTME) is the most recent approach developed to improve pelvic dissection in surgery for mid and low rectal tumours. There are still inconsistencies regarding the technique's long-term oncological results.*

Objectives: *The purpose of this study was to analyse clinical and oncological outcomes of the learning curve of TaTME for mid and low rectal cancer in comparison to a matched cohort of patients treated by laparoscopic total mesorectal excision (lapTME)*

Methods: *Mid and low rectal cancer patients submitted to TaTME and lapTME in two Portuguese colorectal units between March 2016 and December 2018 were eligible. Primary endpoints were 4-year overall survival, disease-free survival and local recurrence. Secondary endpoints were clinical and pathological outcomes.*

Results*: 47 patients underwent TaTME and 44 lapTME. No differences were observed between groups concerning baseline characteristics, emphasizing their comparability. In the TaTME group there were more loop ileostomies performed (33 lapTME versus 44 TaTME, p=0.018) and more hand-sewn anastomosis (0 lapTME versus 7 TaTME, p=0.016), with a trend for lesser distance to the anal verge (35 mm lapTME versus 20 TaTME, p=0.061). There were no differences between groups related to mortality, overall complications, Clavien-Dindo ≥ IIIB morbidity, readmissions and stoma closure. Also, groups were similar in relation to pathological stage, specimen quality, margins, ressecability and node sampling. Finally, no disparities were noted in oncological outcomes, namely local and distant recurrence, 4-year overall survival and 4-year disease-free survival.*

Conclusions: *Even reflecting the learning curve of a new technique, TaTME can be comparable to lapTME, with similar long-term oncological outcomes. It has, however, a demanding learning curve and significant risk for morbidity, for which it should be selectively considered.*

Keywords: rectal cancer, lapTME, TaTME, long-term outcomes, oncological outcomes

RATIONAL AND AIMS

Rational: TaTME has been implemented quite recently for the treatment of rectal cancer. For this reason, the long-term clinical and oncological outcomes are still not clarified. This study hypothesized that TaTME has similar outcomes than classic laparoscopic TME in mid and low rectal cancer.

Aim: Having previously analysed the short and mid-term results, this study's objective was to investigate the long-term clinical and oncological outcomes of the learning curve of TaTME applied exclusively to mid and low RC patients. Also, it intended to compare these outcomes to the ones of a matched group of patients treated by laparoscopic TME.

MATERIALS AND METHODS

This was a retrospective observational study. It compared consecutive patients with mid and low RC stage I-IV, AJCC submitted to TaTME between March 2016 and December 2018 in Hospital Beatriz Angelo and Hospital da Luz in Lisbon with a matched group of patients treated by lapTME in the same institutions. These TaTME patients reflect the learning curve of the technique [316],[317].

The unit's volume of rectal radical resection during the elected study period is shown (Fig 3). Prior to TaTME implementation, surgeons underwent observation of live procedures, hands-on modular training courses and proctored learning. Data regarding TaTME cases was introduced in the International TaTME Registry.

Tumours were defined as in the mid or low rectum if located between 5-10 cm and less than 5 cm from the anal verge, respectively, by magnetic resonance (MR) and rigid sigmoidoscopy.

Patients were selected for TaTME if they presented lesions in the mid or low rectum and all accepted the technique through informed consent.

Pathological specimen plane was defined according to Quirke *et al* as '*muscularis propria* plane', mesorectal plane' or the 'intramesorectal'[7].

In this study, anastomotic leak was defined according to the International Study Group of Rectal Cancer, including radiological and clinical leak, pelvic and perianastomotic abscess [316]. Post-operative morbidity was assessed according to Clavien-Dindo Classification [331] and included all complications related to the initial surgery, even after 30 days. Data was obtained from the hospital's electronic database.

Primary endpoints were oncological outcomes, namely overall survival (OS) disease-free survival (DFS) and local recurrence (LR). Secondary endpoints were clinical, pathological outcomes and parameters of specimen quality.

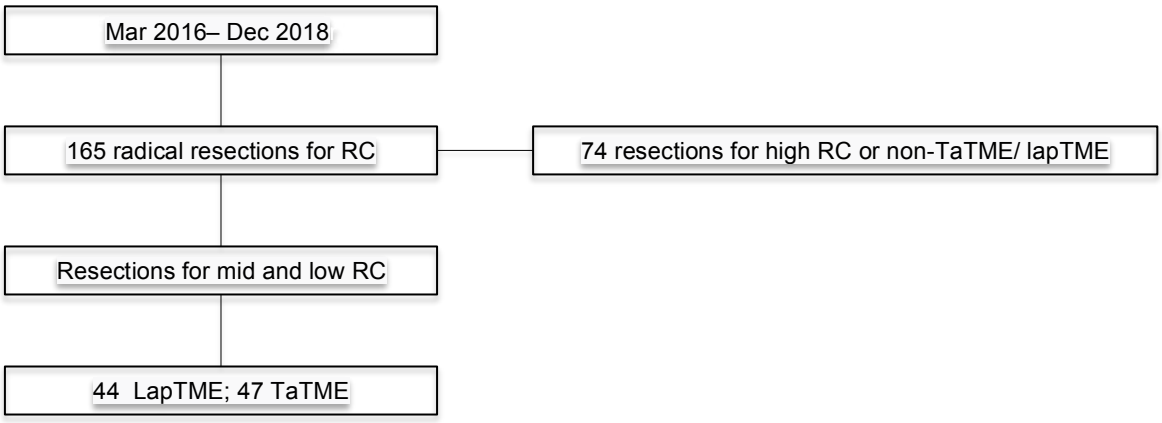


Figure 3 Volume of radical resections for rectal cancer**.** Unit's volume of radical resections (non-exenterative) for rectal cancer (RC) during the elected period of the study. RC rectal cancer, lapTME laparoscopic total mesorectal excision, TaTME transanal total mesorectal excision.

Statistical analysis

In this retrospective study, continuous variables were reported as n, median, first and third quartiles (Q1, Q3). To compare characteristics between patients that performed lapTME or TaTME, independent t test for equal and unequal variances, proportion test, Chi-squared test and Fisher exact test were applied, as appropriate. Analysis time to event data was performed through Kaplan–Meier (KM) curves. Overall survival (OS) was calculated considering surgery date until death date. Disease-free survival (DFS) was estimated considering surgery date until the appearance of recurrence, local or distant. Local recurrence-free survival (LRFS) was assessed measuring time from surgery date till the appearance of LR. Finally, distant progression-free survival (DPFS) was calculated considering surgery date until the appearance of distant progression. Estimated median time to event, 25^{th}- 75^{th} percentiles and correspondent 95% CI were presented. Probability of survival for these time points and respective 95% CI were also disposed. For comparing survival times between groups log-rank test was used. Significance level was set at $p \leq 0.05$. Data was analysed with R (version 4.0.2, 2020-06-22, "Taking Off Again").

RESULTS

Patient clinical parameters

During the elected period, a total of 47 mid and low RC patients were submitted to TaTME and 44 to lapTME with predominance of male gender, PS-ECOG 0 and ASA 2 in both groups. There were no significant differences between groups in terms of baseline characteristics (Table 7).

Table 7 Clinical parameters

Clinical parameters		LapTME (n=44)	TaTME (n=47)	p-value
Gender	Female	14 (31.8)	18 (38.3)	
	Male	30 (68.2)	29 (61.7)	0.518
Age, years, median (range)		69 (59-76)	65 (58-74)	0.693
BMI, kg/ m2, median (range)		26 (24-28)	25 (23- 28)	0.483
	< 25	16 (38)	22 (47)	
	≥ 25	26 (62)	25 (53)	0.407
	ND	2	0	
PS (ECOG), n (%)	0 + 1	37 (90)	45 (96)	
	2 + 3	4 (10)	2 (4)	0.265
	ND	3	NA	
ASA score, n (%)	I + II	24 (65)	32 (71)	
	III +IV	13 (35)	13 (29)	0.545
	ND	7	2	

BMI Body Mass Index, *PS* performance status, *ECOG* Eastern Cooperative Oncology Group, *ASA* American Society of Anaesthesiologists, *TaTME* transanal total mesorectal excision; *LapTME* laparoscopic total mesorectal excision, *NA* not applicable, *ND* not discriminated. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3).

Pre-operative staging and neoadjuvant therapy

The majority of patients were treated for cancer, 46 in the TaTME group and 41 in the lapTME. One patient had TaTME for an endoscopically non-resectable tubulovillous adenoma with high-grade dysplasia and 3 patients underwent lapTME for ulcerative colitis with high-grade dysplasia.

Patients with RC were staged with pelvic MR and thoraco-abdomino-pelvic CT except for 2 that underwent ERUS due to the presence of metallic prosthesis. There were no differences between groups regarding tumour location, extension, distance to the anal verge, cT, cN, cM, clinical stage, CRM, EMVI and CEA.

The majority were patients in stage III, without EMVI and with a free CRM. Likewise, the mainstream of patients in both groups underwent neoadjuvant therapy (32 lapTME versus 23 TaTME,

p=0.686), mostly with a long course chemoradiotherapy (LCCRT). There were no differences between TaTME and lapTME groups regarding tumour characteristics, stage, neoadjuvant regimen chosen and tumour regression grade assessed by MR (mrTRG) (Table 8).

Table 8 Preoperative staging and neoadjuvant therapy

Pre-operative staging and neoadjuvant therapy		LapTME (n=44)	TaTME (n=47)	p-value
Disease (%)	Malignant	41 (93)	46 (98)	
	Pre-malignant	3 (7)	1 (2)	0.350
Location, rectum (%)	1/3 medium	28 (64)	29 (62)	
	1/3 inferior	16 (36)	18 (38)	0.849
Tumour extension (mm), median (range)		50 (31-60)	40 (33-53)	0.596
Distance to anal verge (mm), median (range)		80 (68-90)	70 (50-80)	0.155
cT	T1 + T2	12 (32)	20 (43)	
	T3 + T4	26 (68)	26 (57)	
	NA/ ND	3/ 3	1/ 0	0.264
cN	N0	11 (27)	21 (45)	
	N+	30 (73)	26 (55)	0.082
	NA	3	1	
cM	M0	39 (95)	44 (96)	
	M1	2 (5)	2 (4)	0.999
	NA	3	1	
Stage	Stage I + II	11 (27)	21 (46)	
	Stage III + IV	30 (73)	25 (54)	0.069
	NA	3	1	
CRM, n (%)	Free	26 (74)	35 (76)	
	Threatened or invaded	9 (26)	11 (24)	0.852
	NA/ ND	9	1	
EMVI, n (%)	Negative	26 (74)	35 (76)	
	Positive	9 (26)	11 (24)	0.490
	NA/ ND	3/ 9	1	
CEA (ng/mL)		1.7 (0.7 – 2.6)	1.3 (0.8-2.4)	0.380
CRT	LCCRT	30 (94)	22 (96)	
	SCRT	2 (6)	1 (4)	0.686
	NA	12	24	
mrTRG, n (%)	mTRG 1 + 2	9 (90)	10 (83)	
	mTRG 3	1 (10)	2 (17)	0.999
	NA/ ND	12/ 22	24/ 11	
cCR, n (%)	Negative	28 (88)	20 (87)	
	Positive	4 (12)	3 (13)	0.999
	NA	12	24	

cT cN cM TNM Staging Classification for Rectal Cancer 8th ed., 2017; *CEA* carcinoembryonic antigen, *CRM* circumferential resection margin, *EMVI* extramural vascular invasion, *CRT* chemoradiotherapy, *LCCRT* long course chemoradiotherapy, *SCRT* short course chemoradiotherapy, *mrTRG* magnetic resonance Tumour Regression Grade, *cCR* clinical complete response, *TaTME* transanal total mesorectal excision, *LapTME* laparoscopic total mesorectal excision, *NA* not applicable, *ND* not discriminated. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3). NA in “cT”, “cN”, “cM”, “EMVI” relates to 4 patients with pre-malignant lesions, NA in “CRT”, “mrTRG” and “cCR” relates to patients that did not have neoadjuvant therapy.

Surgical technique

All patients had preoperative mechanical oral bowel preparation and underwent surgical procedure in a median of 12 weeks after chemoradiotherapy (CRT) (range 10-13 and 11-13, p=0.266 in TaTME and lapTME groups, respectively).

The TaTME procedure was performed with 2 teams, transabdominal and transanal, working synchronously, with complete mobilization of the splenic flexure in all cases, using *Lone Star® Retractor (Cooper Surgical, USA*) and *GelPOINT®Path Transanal Access Platform (Applied Medical, USA)* for the transanal approach. This procedure was done through laparoscopy in 39 (82%) patients and robotically in 4 (9%) cases, in a total of 43 (91%) through minimally invasive approach. With no transanal conversions, there were 4 (9%) abdominal conversions to midline laparotomy, 1 due to pre-sacral bleeding, 1 for pneumoperitoneum intolerance, and 2 for obesity-related technical difficulties. Concurrently with the protectomy, 4 protocolectomies and 2 liver metastasis resections were made, also by laparoscopy.

There were no differences between groups related to the number of anastomosis performed, with a predominance of mechanical, side-to-end anastomosis in both. Cohorts were comparable regarding specimen extraction site, intraoperative blood lost, complications and operative time. There were, however, more hand-sewn anastomosis in TaTME group (0 lapTME *versus* 7 TaTME, p=0.016) with a trend for a lesser distance from the anal verge (35 mm lapTME *versus* 20 TaTME, p=0.061). Also, more loop-ileostomies (33 LapTME versus 44 TaTME, p=0.018) were used in the TaTME group. On the contrary, more pelvic drains were placed in the lapTME cohort (30 lapTME *versus* 22 TaTME, p= 0.039) (Table 9).

Post-operative period and Follow-up

There were no differences between groups in 30-day mortality, overall complications rate and Clavien-Dindo morbidity higher than IIIB (18% lapTME *versus* 23% TaTME p=0.859). Being defined as *per* the International Study Group of Rectal Cancer [316], anastomotic leakage rate was not different between cohorts (11% lapTME *versus* 17% TaTME, p= 0.367). Of these leaks, in the lapTME group, 4 patients underwent surgical re-exploration with 2 end colostomies, one transabdominal and one transanal drainage. In the TaTME group, 6 patients had to be re-operated with one end colostomy, one trans-abdominal and 4 transanal drainages. Overall, 36 (95%) and 43 (98%) patients maintained their anastomosis in the lapTME and TaTME groups, respectively (Table 10).

No differences were found regarding length of hospital stay, readmission rate, stoma closure and number of patients undergoing adjuvant therapy. Until the final date of this study, 29 (88%) and 37 (84%) had their ileostomies closed in the lapTME and TaTME groups, respectively (Table 10).

Table 9 Surgical technique

Surgical Technique		LapTME (n= 44)	TaTME (n= 47)	p-value
Type of surgery, n (%)	Anterior resection	8 (18)	0 (0)	
	Low anterior resection	36 (82)	0 (0)	<0.001*
	TaTME	0 (0)	47 (100)	
CRT- surgery, weeks, median (range)		12 (10-13)	12 (11-13)	0.266
Abdominal approach, n (%)	Laparoscopy	44 (100)	39 (82)	
	Laparotomy	NA	4 (9)	0.006*
	Robotic	NA	4 (9)	
Anastomosis, n (%)	Yes	38 (86)	44 (94)	
	No	6 (14)	3 (6)	0.250
Anastomosis, type, n (%)	Mechanical	35 (100)	37 (84)	
	Hand-sewn	0 (0)	7 (16)	0.016*
	ND	3	0	
Anastomosis, type, n (%)	Side-to-end	19 (63)	24 (68)	
	End-to-end	8 (27)	10 (29)	
	Ileoanal pouch –anal	3 (10)	1 (3)	0.596
	ND /NA	8/ 6	9/ 3	
Anastomosis distance from dentate line, mm, median (range)		35 (18-60)	20 (10-40)	0.061
Specimen extraction site, n (%)	LIF	12 (43)	8 (19)	
	Pfannenstiel	16 (57)	33 (76)	
	Transanal	0 (0)	2 (5)	0.051
	ND/ NA	16/ 0	0/ 4	
Operative morbidity, n (%)		0 (0)	3 (6)	
Abdominal approach	Pre sacral bleeding	0	1	
Transanal approach	Vaginal lesion	0	1	0.242
	Urethral lesion	0	1	
Loop ileostomy, n (%)	Yes	33 (87)	44 (100)	
	No	5 (13)	0	0.018*
	NA	6	3	
Drains, n (%)	Yes	30 (68)	22 (47)	
	No	14 (32)	25 (53)	0.039*
Blood lost, mL, median (range)		150 (100-250)	200 (100-300)	0.226
Operative time, min, median (range)		290 (245-338)	285 (255-340)	0.965

CRT chemoradiotherapy, *LIF* left iliac fossa, *min* minutes, *NA* not applicable, *IPAA* ileal pouch anal anastomosis, *TaTME* transanal total mesorectal excision, *LapTME* laparoscopic total mesorectal excision, *ND* not discriminated, *NA* not applicable. Continuous variables are reported as n, median, range of first and third quartiles (Q1-Q3), *p-value < 0.05. NA in "Anastomosis type" and "Loop ileostomy" relates to 9 patients that did not have an anastomosis, NA in "Specimen extraction site" relates to 4 TaTME patients that were converted to laparotomy.

Table 10 Post-operative period and Follow-up

Post-operative period	LapTME (n=44)	TaTME (n=47)	p-value
Admission (days), median	7 (5-14)	7 (4-14)	0.992
30-day mortality, n (%)	2 (5)	0 (0)	0.109
Readmission, n (%)	3 (7)	5 (11)	0.719
Postoperative complications (treatment), n (%)			
Clavien-Dindo < IIIB	13 (30)	8 (17)	
Clavien-Dindo ≥ IIIB	10 (23)	11 (23)	0.859
Clavien-Dindo I	6 (14)	1 (2)	
Clavien-Dindo II	7 (16)	7 (15)	
Clavien-Dindo IIIB	6 (14)	11 (23)	
Abdominal wall dehiscence (closure abdominal wall)	-	1	
Intra abdominal bleeding (ligation of epigastric vessels)	1	-	
Pancreatic fistulae (drainage)	-	1	
Intra-abdominal haematoma/ collection (drainage)	1	1	
Parastomal hernia (suture)	-	1	
Internal hernia (reduction)	1	-	0.083
Small bowel injury (enterorraphy)	-	1	
Necrosis of colostomy (segmental resection)	1	-	
Anastomotic leak			
(Transanal drainage)	1	4	
(Transabdominal drainage)	-	1	
(End colostomy)	1	1	
Clavien-Dindo V	2 (4)	0 (0)	
Anastomotic leak (colostomy)	2	-	0.109
Overall Leak, n (%)	4 (11)	7(17)	0.367
Ileostomy closure, n (%)			
Yes	29 (88)	37 (84)	
No	4 (12)	7 (16)	0.749
NA	11	3	
Adjuvant CT, n (%)			
Yes	23 (56)	23 (50)	
No	18 (44)	23 (50)	0.668
NA	3	1	

Leak defined according to International Rectal cancer Study Group, Complications classified according to Clavien-Dindo classification, *AB* antibiotic treatment, *CT* chemotherapy, *CLD* chronic liver disease. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3).

Pathological Outcomes

There were no differences between groups related to pathological stage, circumferential, proximal and distal margins, ressectability, node sampling and specimen quality. Both techniques showed good quality specimens with appropriate margins and lymphadenectomies (Table 11).

Table 11 Pathological Outcomes

	LapTME (n=44)	TaTME (n=47)	p-value
Stage, n (%)			
TxN0M0	7 (18)	4 (9)	
I	15 (37)	21 (46)	
II	6 (15)	5 (11)	0.601
III	10 (25)	15 (32)	
IV	2 (5)	1 (2)	
ND/ NA (high grade dysplasia)	4	1	
Ressectability, n (%)			
R0	43 (98)	47 (100)	0.309
R1	1 (2)	0 (0)	
Mesorectal plane, n (%)			
Mesorectal/ intramesorectal plane	34 (94)	45 (96)	
Muscularis propria plane	2 (6)	2 (4)	0.999
ND	8	0	
Proximal Margin, n (%)			
Free	44 (100)	47 (100)	NA
Invaded	0	0	
Distal Margin, n (%)			
Free	44 (100)	47 (100)	NA
Invaded	0	0	
CRM, n (%)			
Free	42 (96)	47 (100)	0.191
Threatened/ Invaded	2 (4)	0	
Distal Margin, mm, median (range)	25 (15-30)	20 (10-25)	0.382
Nodes, median (range)	14 (9-20)	19 (12-24)	0.649

Pathological Staging for according to the American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017, Specimen quality/ mesorectal plane classified according to P. Quirke, *NA* not applicable, *ND* not discriminated. Continuous variables are reported as median, range of first and third quartiles (Q1, Q3).

Oncological outcomes

Because the majority of patients in the TaTME group only achieved 4 years of follow-up, we report 4-year oncological outcomes.

In the lapTME group there was one (2%) case of LR at 16 months, in the pre-sacral area in a patient with previous distant disease. There were 4 (10%) cases of distant progression (DP) after a median of 15 (6-23) months in patients that were initially stage III (3 cases) and IV (1case). In this cohort there were 7 (16%) deaths, 1 due to disease progression, 2 to complications of index surgery and 4 to non-oncological co-morbidities (vascular, liver and cardiac insufficiency) (Table 6). Four-year OS and DFS were 82% (CI 0.713-0.953) and 91% (CI 0.825-1), respectively. Also, 4-year DPFS and LRFS were 91% (CI 0.825-1) and 96% (CI 0.882-1), correspondingly (Fig. 4, Table 12).

In the TaTME group there were 2 (4%) cases of LR at 8 and 22 months. Recurrences were presacral and anastomotic, respectively, with no pelvic sidewall pattern. The patient with a pre-sacral recurrence had synchronous hepatic metastasis and a suboptimal specimen with an incomplete mesorectum following a procedure with long operative time and an intraoperative urethral lesion. In this cohort there were 10 (21%) cases of distant disease, one synchronous with LR and 9 metachronous, after a median of 8 (7-11) months. Patients who developed distant metastasis were initially in stage IV in 2 cases and stage III in 7. Metastatic disease involved the lung, liver, central nervous system, bone and peri-aortic nodes. In the TaTME group there 5 deaths, all related to distant disease progression (Table 6). Four-year OS and DFS were 86% (CI 0.760-0.985) and 78% (CI 0.666-0.910), respectively. Finally, 4-year DPFS and LRFS were 78% (CI 0.666-0.91) and 94% (CI 0.860-1), correspondingly (Fig. 4, Table 12).

Overall, there were no differences between lapTME and TaTME groups related to mortality (p=0.543), LR (p=0.999) and DP (p=0.158). Likewise, cohorts presented similar 4-year OS, DFS, LRFS and DPFS (p=0.4, p=0.1, p=0.7 and p= 0.1 respectively) (Fig. 4, Table 12).

Table 12 Oncological Outcomes

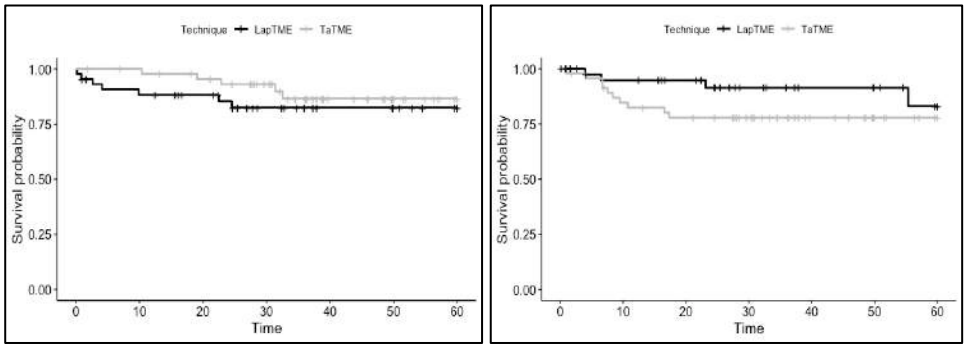
	LapTME	TaTME	p-value
Follow up time (months), median	33 (17-56)	36 (28-48)	0.464
Local recurrence, n (%)	1 (2)	2 (4)	0.999
Presacral	1	1	
Anastomotic	-	1	
Distant progression	4 (10)	10 (21)	0.158
Lung	2	3	
Liver	2	3	
CNS + bone	-	2	
Periaortic nodes	-	1	
Inguinal node	-	1	
Global Mortality, n	7 (16)	5 (11)	0.543
4y OS probability, %, CI	82 (CI 0.713-0.953)	86 (0.760-0.985)	0.4
4y DFS probability, %, CI	91 (CI 0.825-1)	78 (CI 0.666-0.91)	0.1
4y DPFS probability, %, CI	91 (CI 0.825-1)	78 (CI 0.666-0.91)	0.1
4y LRFS probability, %, CI	96 (CI 0.882-1)	94 (CI 0.860-1)	0.7

CNS central nervous system, OS overall survival, DFS disease free survival, LRFS local recurrence free survival, DPFS distant progression free survival, CI confidence interval. Continuous variables are reported as median, range of first and third quartiles (Q1, Q3).

Figure 4 Oncological Outcomes

a) Overall survival

b) Disease-free survival



c) Local recurrence-free survival

d) Distant progression-free survival

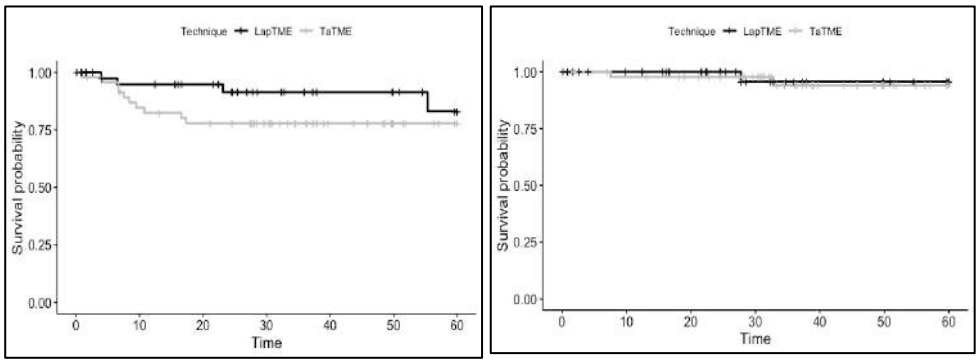


Fig 4 Kaplan–Meier curves for a) Overall survival (OS), b) Disease-free survival (DFS), c) Local recurrence-free survival (LRFS) and d) Distant progression-free survival (DPFS) according to the technique, TaTME: transanal total mesorectal excision, LapTME laparoscopic total mesorectal excision. There were no differences between groups related to 4-year OS (p=0.4), DFS (p=0.1), LRFS (p=0.7) and DPFS (p= 0.1).
(a) LapTME 4-year OS was 82% (CI 0.713-0.953), b) 4-year DFS was 91% (CI 0.825-1), c) 4-year LRFS was 96% (CI 0.882-1) d) 4-year DPFS was 91% (CI 0.825-1)
(a) TaTME 4-year OS was 86% (0.760-0.985), (b) 4-year DFS was 78% (CI 0.666-0.910), (c) 4-year LRFS was 94% (0.860-1), d) 4-year DPFD was 78% (CI 0.666-0.910).

DISCUSSION

Despite the great advance in rectal surgery brought by lapTME in terms of short and long-term outcomes, this technique can be very demanding, particularly in a specific group of patients with obesity and distal bulky tumours. LapTME for mid and low RC has been associated with high anastomotic leak, conversion and suboptimal TME specimens, with known deleterious oncological consequences [10], [113],[332],[333]. The difficulty relates to operating in the low pelvic compartment with restricted working space, limited vision and manoeuvrability.

Surgeons have tried to developed alternatives to overcome these problems and TaTME was introduced in 2010 to improve pelvic approach [327],[330]. Having previously analysed the short and mid term results of the learning curve of TaTME in our institution [328], the present study had the objective of

analysing the long-term clinical and oncological outcomes. It also projected the comparison of these outcomes to the ones of a matched group of patients treated with lapTME by the same surgeons.

In this study TaTME and lapTME groups were comparable in terms of demographic and clinical characteristics, with no differences in terms of gender, age, BMI, PS, ASA scores, baseline tumour characteristics, neoadjuvant therapy and subsequent response. Groups were also surgically comparable with the exception that TaTME patients had more hand-sewn anastomosis (0 lapTME *versus* 7 TaTME, p= 0.016) and loop ileostomies performed (33 lapTME *versus* 44 TaTME, p= 0.018). LapTME had more drains placed in the pelvis (30 lapTME *versus* 22 TaTME, p= 0.039).

So far, published literature show that TaTME has short-term clinical outcomes similar or better than lapTME regarding conversion, anastomotic leak, distal and circumferential margins, mesorectal integrity, lymph node yield, operative time, blood lost, morbidity, length of hospital stay (LOS) and readmission rates [294], [301]-[303],[322].

In our work, we also obtained similar outcomes regarding LOS, re-admission rates, overall complications, morbidity higher than Clavien-Dindo IIIB and overall leak rate. Although we report that 8 patients in the lapTME group had to be re-operated, anastomotic leak rate was 11%. Likewise, in the TaTME cohort, while 11 patients had a re-intervention, only 6 were due to anastomotic leak. Although not statistically different between cohorts (11% lapTME *versus* 17% TaTME, p= 0.367), the anastomotic leak rate in TaTME group, probably a consequence of the learning curve, is worrisome and must be mitigated. Regarding pathological outcomes, there were no disparities between groups in stage, ressectability, node sampling, circumferential, proximal and distal margins and specimen quality.

Although short-term clinical outcomes seem to be well established, contradictions remain regarding oncological outcomes and some authors have even reported disturbing results of early sidewall and multifocal pelvic cavity recurrence. In this work, we did not experience these negative outcomes, which was possibly a consequence of several reasons, such as the use of a non-standardized procedure, surgeons endorsing TaTME prior to a proficient learning curve or even slight technical differences between surgical teams. In fact, we still cannot fully comprehend the discrepancy of results between publications.

So far, very few studies that report on TaTME have a follow-up period longer than 3 years. Marks *et al* analysed 373 patients submitted to TATA with the abdominal dissection done through pure transanal, laparoscopic, robotic or open approach. With 66 (range 0–300) months of mean follow-up, 5-year LR was 7.4% and OS was 90% [313],[329]. Recently, Hol *et al* reported on 159 TaTME patients with 5 year 4% LR, 77% OS and 81% DFS [334]. Lastly, in a trial with 100 patients randomly assigned to TaTME and lapTME, there was no difference in 5-year LR or DFS between groups [335]. The fact that most other studies only report short-term outcomes has not permitted definitive conclusions.

In our study, the lapTME group had one (2%) case of LR, happening at 16 months, in the pre-sacral area in a patient with prior distant progression. In the TaTME group there were 2 (4%) cases of LR, pre sacral and anastomotic, none multifocal or in the pelvic sidewall. Overall, no differences were perceived regarding LR (p=0.999). In the lapTME group, 4-year OS and DFS were 82% (CI 0.713-0.953) and 91% (CI 0.825-1) similar to the 86% (CI 0.760-0.985) and 78% (CI 0.666-0.910), respectively, presented by the TaTME group. Also, no differences between groups occurred in 4-year OS, DFS, LRFS and DPFS (p=0.4, p=0.1, p=0.7 and p= 0.1 respectively).

The main limitation of this work is its non-randomized methodology. Notwithstanding, the similarity observed between groups in respect to baseline characteristics emphasizes their comparability. Also, the follow-up of this study is longer than what most studies published so far.

Our results show similar pathological and oncological outcomes between lapTME and TaTME, in accordance to what has been the generalised perception of the technique. Having said this, they also show how demanding this new technique can be and consequently the need for audited data and standardized implementation.

CONCLUSION OF BOTH STUDIES

Intended to show the outcomes of the learning curve of TaTME in our Colorectal Group [337]-[339], both these studies showed that the technique can produce short and long-term oncological safe outcomes. Also, results were similar to our matched lapTME cohort and compatible to what has been published for the laparoscopic technique for mid and low RC.

It must be emphasized, however, that TaTME has a demanding learning curve and significant risk for morbidity. For its safe introduction it is fundamental to understand the different anatomical perspective it involves [340]-[343], implement intensive multimodal learning with hands-on cadaver training and proctoring, follow international guidelines and selectively apply the technique [114],[123],[343],[344]. Also, it is imperative that surgeons are experienced not just in laparoscopy but also in single-port and low pelvic surgery.

TaTME cannot be seen as a technique to replace either laparoscopic or open approaches but rather as another option available in the surgical armamentarium, indicated in particular cases, mainly obese male patients with distal tumours. We still cannot fully comprehend the disparity of results between publications regarding oncological outcomes. In this context, it becomes imperative to contribute to a better understanding of this new technique by reporting one's results.

Original Paper 6

Oncological Outcomes of Local Excision Compared with Radical Surgery after Neoadjuvant Chemoradiotherapy for Rectal Cancer: A systematic review and metanalysis

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ABSTRACT

***Background**: Locally advanced rectal cancer is conventionally managed with neo-adjuvant chemoradiotherapy (CRT) followed by radical surgery. In patients who refuse a stoma or are unfit for radical surgery an alternative approach may be the use of neoadjuvant CRT and local excision (LE) where tumours are responsive.*

***Objectives**: The aim of this systematic review is to determine whether differences exist in local recurrence, overall and disease free survival between patients treated with CRT+LE and those with CRT+ radical surgery (RS) for rectal cancer.*

***Methods**: A literature search was performed using MEDLINE, PubMed and Ovid databases and Google Scholar. Studies comparing outcome following LE and RS post CRT were included. A pooled analysis was carried out using the Mantel-Haenszel statistical model to identify differences in local recurrence (LR), Overall Survival (OS) and Disease Free Survival (DFS), between treatment strategies.*

***Results**: A total of eight studies met the inclusion criteria. All studies were suitable for pooled analyses of LR whereas five studies contributed to analyses for OS and four for DFS. When RS was used as the reference group, there was no significant difference between the risk of LR between LE and RS groups (OR: 1.39, CI; 0.78-2.47, p=0.26). Similarly, no difference was observed in 10 year OS (OR 0.98, CI; 0.41-2.34, p=0.96) or 5-year DFS (OR 0.80, CI; 0.41-1.56, p=0.52). There was evidence of publication bias amongst the publications included in analyses of DFS. Subgroup analysis of treatment benefit between the two treatment modalities for T3/ any N stage cancers showed no statistical difference in outcome measures.*

***Conclusion**: There was no difference in the LR, OS or DFS between patients treated with LE and those undergoing RS of rectal cancer following neoadjuvant CRT. LE post CRT represents a viable alternative to RS for some patients wishing to avoid radical surgery.*

Keywords: low rectal cancer; chemoradiotherapy; local excision; TEMS; anterior resection

INTRODUCTION

Locally advanced rectal cancer (LARC) is conventionally treated with CRT followed by radical surgery (RS). Current guidelines recommend that low risk RC (T1-T2 and T3a with N0) be managed by surgery alone and moderate to high-risk tumours (over T3b with threatened CRM or encroaching into intersphincteric plane/ levator muscle plate) are recommended for LCCRT or SCRT [17]. Radical surgery includes TME with anterior resection or abdominoperineal excision, performed by laparoscopic, transanal, robotics or open approach.

TME is, however, a major surgical procedure with a 30-day mortality rate of 0.9-1.5% and significant morbidity (38.0- 54.0%), including anastomotic leakage, injury to genitourinary nerves as well as variable bowel functional outcome [123],[345]. In reality, 50-80% patients have some form of LARS with reduced quality of life, 32-80% present sexual or bladder dysfunction and 10-20% require either a temporary or permanent stoma [346]. Also, TME has an expected 5-year LR and OS of 6-8% and 76%, respectively [153],[347],[348].

This situation has prompted the search for less aggressive therapeutic strategies to reduce the morbidity associated with TME such as a neoadjuvant therapy followed by organ-preserving procedures, like local excision (LE) or the Watch and Wait (WW) approach [349].

The introduction of neoadjuvant CRT in RC management has led to significant improvements in local disease control with frequent tumour downsizing, downstaging or disappearance, and sterilization of micrometastasis in lymph nodes. In this setting, transanal full-thickness LE has been considered for the management of selected patients with significant response to CRT. Endoscopic assessment and MR restaging have been shown to be sensitive tools that facilitate the selection of suitable patients [350].

The concern regarding LE is that it may be associated with a greater risk of recurrence because a smaller amount of tissue is excised and regional lymph nodes are not removed.

In the post CRT context, LE is currently performed in two scenarios: 1) in very good but incomplete responses with tumour confined to the rectal wall (ycT1) or 2) as an alternative to radical surgery in medical inoperable patients due to age or co-morbidities, if there is an indication for APER or difficult intersphincteric resection (with total or subtotal internal sphincter resection) or in those that refuse radical surgery. Overall, these cases represent no more than 10-15% of all patients with low and mid rectal cancer.

RATIONAL AND AIMS

Rational: Considering the morbidity associated with radical surgery for RC, less aggressive strategies have been considered. This study hypothesized that QRT followed by LE could have the same oncological outcomes that QRT with radical excision in specific group of patients,

Aims: A review of the available literature on LE performed after CRT was conducted to define the state of the art of this conservative approach including controversial aspects concerning this subject, in particular tumour scatter, lymph node status, completion and salvage surgeries and LE related morbidity. The aim of this study was to compare local recurrence (LR), Overall Survival (OS) and Disease Free Survival (DFS) rates between Local Excision (LE) and Radical Surgery (RS) for rectal cancer, in the post CRT setting. Secondary aim was to analyse the differences in outcomes depending on specific tumour stage.

MATERIAL AND METHODS

A literature search was performed using MEDLINE, PubMed database, Ovid and Google Scholar. The following keywords were used; rectal cancer (RC) combined with surgical resection, local excision (LE) and total mesorectal excision, abdominoperineal resection, radical surgery (RS), without any restriction on language. Searching was restricted to human studies. In instances where there was more than one publication by the same investigating group using the same study population, the latest study was used unless studies referred to different patient populations. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses - PRISMA flow diagram was used to show the search methodology.

Inclusion Criteria

Studies were included if they investigated LR, OS and DFS in patients with rectal adenocarcinoma with any T stage and N1 status. Only studies that involved a direct comparison between LE and RS after CRT were included.

Exclusion Criteria

Studies that did not describe the above outcomes, reviews, editorials or where there was insufficient information provided for data extraction were excluded. Similarly, studies involving patients undergoing surgery for recurrent disease or that included patients with metastatic disease from the outset were also excluded.

Data Extraction and quality assessment

Data was extracted by the investigators using a predefined proforma. Data was collected on patients undergoing local and radical resection and investigated the primary outcomes of disease recurrence, OS and DFS. Disagreements were resolved by discussion with the third investigator. Where data extraction was not possible due to insufficient information, the study was excluded. The NICE for Quality Assessment of Case Series was used to evaluate the quality of studies (NICE, www.nice.org.uk).

Statistical Method and Publication Bias

All data were analysed using Review Manager 5 (RevMan, versions 5.2.1, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). The Mantel-Haenszel method, using random effects analysis, was used to evaluate risk (odds ratio) of cancer recurrence, OS and DFS between the two groups (LE and RS). A funnel plot applying Egger's test was charted to evaluate the risk of publication bias amongst the included studies. Funnel plots were used to determine potential risk of publication bias. In the studies selected for LR and DFS, there were no outliers beyond the 95 % CI margins, suggesting little risk of bias.

RESULTS

The search retrieved a total of 84 articles (Fig. 5). Three further articles were identified through manual searching. Duplicates were removed and review articles were excluded, leaving a total of 66 abstracts for screening. Within these, 50 articles were excluded, as the studies did not provide specific data on surgical or oncological outcome, leaving a total of 16 articles. Of these, a further eight were excluded, as they did not compare LE with RS, leaving a total of eight articles [151], [348]–[354]. Of these eight (Table 13), seven had pooled their recurrence and survival outcomes across different tumour stages. Further subgroup analysis for LR of T3 (any N stage) cancers resulted in only two studies and no isolated outcomes of interest were reported for T1 or T2 stage. Only one study [352] was a randomized controlled trial (RCT) and was therefore reported separately and not included in meta-analysis.

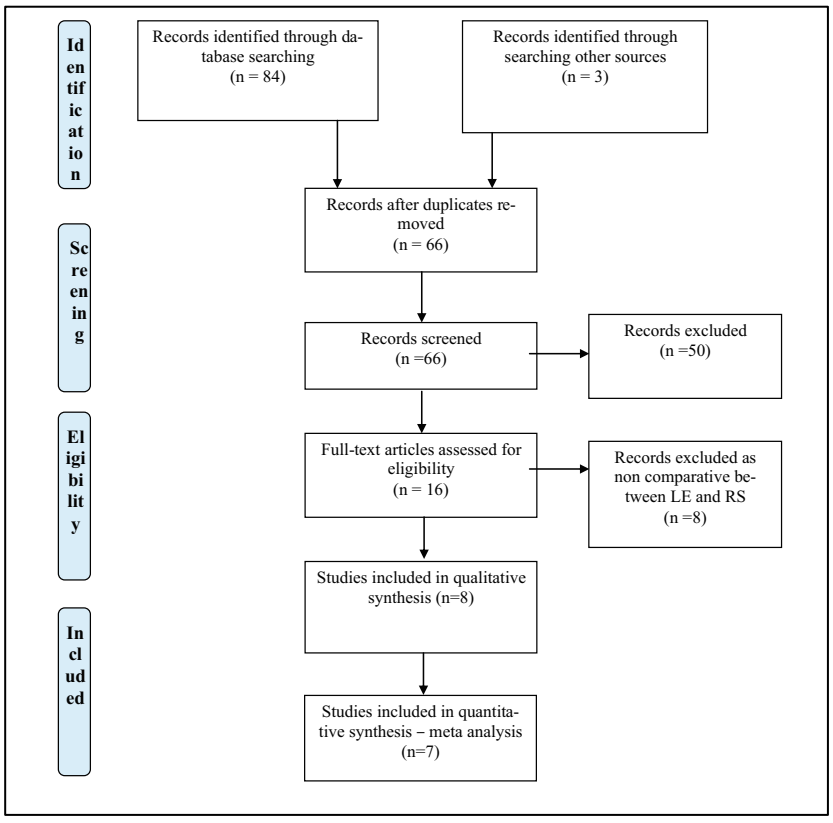

Figure 5 A PRISMA diagram outlining and search strategy and selections of included studies

Table 13 Study characteristics and patient demography

Study (year)	Type of study	Study period	Country	LE group (*n*)	RS group (*n*)	Sex (*n*)		Mean age (range)		Median follow-up in months (range)	
						LE	RS	LE	RS	LE	RS
Bannon et al. [14] (1995)	Prospective cohort	1993–1995	USA	44	65	M, 22 (50 %) F, 22 (50 %)	M, 43 (66.2 %) F, 22 (33.8 %)	68 (42–86)	59 (29–83)	36 (2–94)	40 (1–107)
Bonnen et al. [15] (2004)[a]	Prospective data collection; retrospective analysis	1990–2002	USA	26	405	M, 17 (65 %) F, 9 (35 %)	M, 247 (61 %) F, 158 (39 %)	60 (35–80)	59 (21–88)	42 (4.5–109)	32 (3–113)
Callender et al. [16] (2010)	Retrospective cohort	190–2008	USA	47	473	–	–	62.5 (48.3–76.7)	58.3 (45.8–70.3)	63 (9–178)	59 (4–172)
Caricato et al. [17] (2006)[a,b]	Prospective cohort	1997–2002	Italy	8	22	M, 22 (73.4 %) F, 8 (26.6 %)	64 (49–75)	37 (24–66)			
Habr-Gama et al. [18] (1998)	Prospective cohort	1991–1996	Brazil	9	78	M, 68 (57.6 %) F, 50 (42.4 %)	57 (21–82)	36 (8–67)			
Huh et al. [19] (2008)[a,b]	Retrospective cohort	1994–2005	South Korea	9	64	M, 5 (55.6 %) F, 4 (44.4 %)	M, 41 (64.1 %) F, 23 (35.9 %)	55 (42–69)	54 (31–80)	91 (50–127)	55 (3–132)
Kundel et al. [20] (2010)[a,b]	Retrospective cohort	1997–2007	Israel	14	37	M, 160 (50 %) F, 160 (50 %)	70 (60–79)	68 (40–78)	67 (28–86)	48 (5–123)	
Lezoche et al. [11] (2012)[a,b]	Randomised control trial	1997–2004	UK	50	50	M, 30 (60 %) F, 20 (40 %)	M, 34 (68 %) F, 16 (32 %)	66 (58–70)	66 (60–69)	115 (102–133)	115 (89–143)

LE local excision, *RS* radical surgery, *M* male, *F* female

[a] Studies included in 5-year overall survival (OS)

[b] Studies included in 10-year disease-free survival (DFS)

Table 14 Outcomes

Study (year)	Tumour stage before CRT (*n*)		Tumour stage after CRT (*n*)		Local recurrence						Number of patients survived disease free (5-year DFS)				Number of patients survived overall (10-year OS)			
	LE	RS	LE	RS	LE group (*n*)	RS group (*n*)	LE (*n*)	LE rate	RS (*n*)	RS rate	LE DFS (*n*)	LE DFS rate	RS DFS (*n*)	RS DFS rate	LE OS (*n*)	LE OS rate	RS OS (*n*)	RS OS rate
Bannon et al. [14] (1995)	T0, 1 T1, 5 T2, 6 T3, 2	T0/N0, 10 T1/N0, 2 T2/N0, 23 T0/N1, 1 T2/N1, 8 T3/N0, 11 T3/N1, 6 T3/N2, 4	–	–	44	65	6	13.6 %	6	9.2 %	–	–	–	–	40	90.9 %	52	80.0 %
Bonnen et al. [15] (2004)[a]	T3/N0, 25 T3/N1, 1	T3/N0, 176 T3/N1, 229	–	–	26	405	2	7.7 %	32	7.9 %	19	73.1 %	328	81.0 %	–	–	–	–
Callender et al. [16] (2010)	T3	T3	–	–	47	473	5	10.6 %	36	7.6 %	–	–	–	–	–	–	–	–
Caricato et al. [17] (2006)[a,b]	T2/N0, 5 T2/N1+, 3 T3/N0, 3 T3/N+, 14 T4/N0, 2 T4/N+, 20	T2/N0, 8	–	8	22	0	0.0 %	3	13.6 %	8	100.0 %	17	77.3 %	–	–	–	–	–
Habr-Gama et al. [18] (1998)	T1/N0, 11 T2/N0, 29 T3/N0, 38	T0/N0, 6 T1/N0, 39 T2/N0, 18 T3/N0, 14 TxNx, 1	9	78	2	22.2 %	9	11.5 %	–	–	–	–	–	–	–	–	–	–
Huh et al. [19] (2008)[a,b]	T2/N0, 1 T3/N0, 5 T3/N1, 3	–	–	–	9	64	1	11.1 %	5	7.8 %	7	77.8 %	40	62.5 %	8	88.9 %	48	75.0 %
Kundel et al. [20] (2010)[a,b]	T2/N0, 3 T3/N0, 7 T1–2/N1, 1 T3–4/N1, 3	T2/N0, 9 T3/N0, 21 T1–2/N1, 1 T3–4/N1, 6	T0/N0, 6 T1–2/N0, 6 T3/N0, 2 T1–T2/N1, 0 T3–T4/N1, 0	T0/N0, 13 T1–2/N0, 7 T3/N0, 6 T1–T2/N1, 8 T3–T4/N1, 3	14	37	0	0.0 %	3	8.1 %	14	100.0 %	33	89.2 %	14	100.0 %	36	97.3 %
Lezoche et al. [11] (2012)[a,b]	–	–	T0, 14 T1, 12	T0, 13 T1, 12	50	50	4	8.0 %	0	0.0 %	45	90.0 %	47	94.0 %	40	80.0 %	43	86.0 %

LE local excision, RS radical surgery CRT chemoradiotherapy, a) studies included in 5-year overall survival (OS); b) studies included in 10-year disease-free survival (DFS)

Local Recurrence

Of the seven included studies eligible for LR analyses, three studies were from the USA, the others being from Italy, Brazil, South Korea and Israel. The total patient population in the pooled analysis was 1301 with 157 patients in the LE group and 1144 patients in the RS group. Across the seven studies, four observed a higher recurrence rate in the LE group, while three observed a higher rate of recurrence in the RS group (Table 14). The RCT reported LR in four patients (4/50, 8%) in the LE group, compared with three (3/50, 6%) in the RS group. Pooling of data excluding the RCT (Fig. 6) from relevant studies demonstrated that a total of 16 patients (16/157, 10.1%) had LR in the LE group and 95 (95/1,144, 8.0%) in the RS group. The pooled OR of LR was 1.29 (CI 0.72-2.31, p=0.40) with no difference between groups. There was no heterogeneity in the pooled analysis (I^2=0 %). Subgroup analysis (Fig. 7) of the studies [353],[354] investigating T3 and any N stage cancers revealed no significant difference (OR 1.28, CI 0.56–2.91, p=0.56) in LR rates between LE (7/73, 9.5%) and RS (68/ 878, 7.7%).

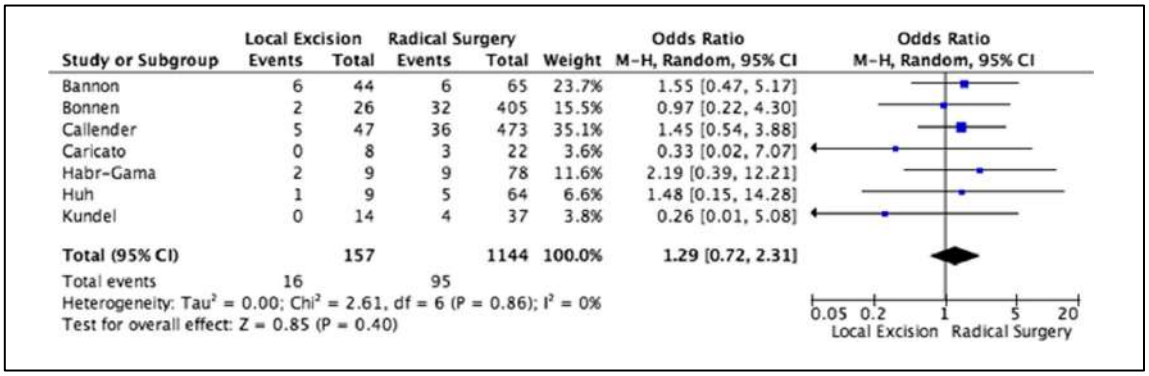

Figure 6 Local recurrence across all stages

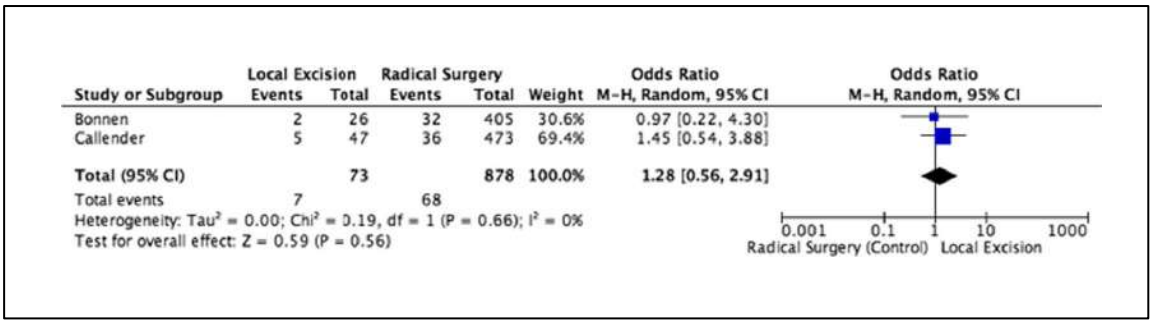

Figure 7 Local recurrence for T3 tumours only

Disease-free Survival

Five studies provided data on 5-year DFS for pooled analysis. The total population in this subgroup was 1105 patients (LE, n=127; RS, n=978). Pooled results (Fig. 8) did not demonstrate a difference in DFS between the RS and LE groups (OR 1.04, CI 0.61–1.76, p=0.89). Equally, the RCT also did not show a difference in DFS between the RS and LE patient groups (p=0.686). Subgroup analysis of tumours equal or greater than cT3 also showed no difference in DFS (OR 0.73, CI 0.43–1.24, p=0.24) (Fig. 9).

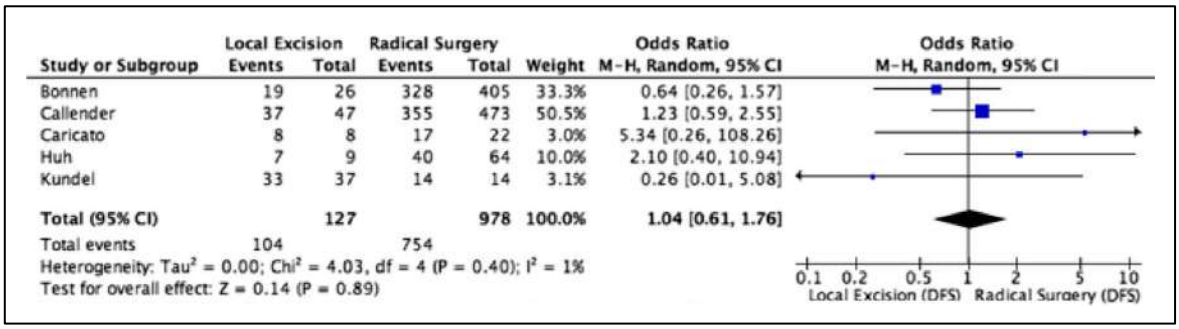

Figure 8 Disease-free survival across all stages

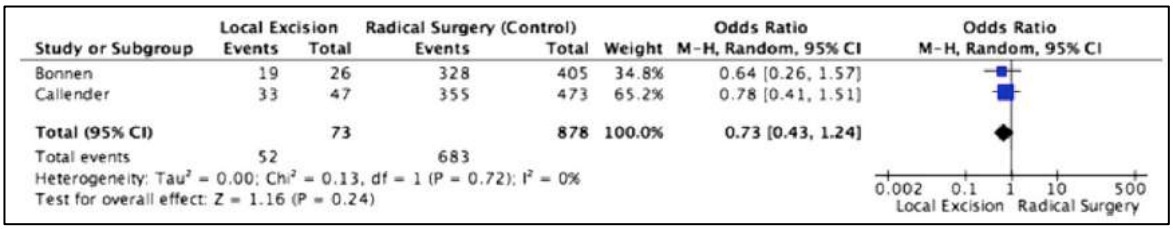

Figure 9 Disease-free survival for T3 rectal tumours

Overall Survival

Four studies were selected for 10-year survival pooled analysis. The total population included in the analysis was 585 patients (LE, n=80; RS, n=505). The 10- year OS (Fig. 8) in the LE group was 83.5% (67/80) and 79.0% (399/505) in the RS group. All studies showed better survival in the LE group but failed to reach statistical significance. The RCT showed no significant difference in OS in LE versus RS (p=0.609). Pooled analysis did not demonstrate a difference in OS between LE and RS (OR 0.96, CI 0.38–2.43, p=0.93) (Fig.10) Further subgroup analysis for tumours equal or greater than cT3 was not possible as only one study reported the required outcome.

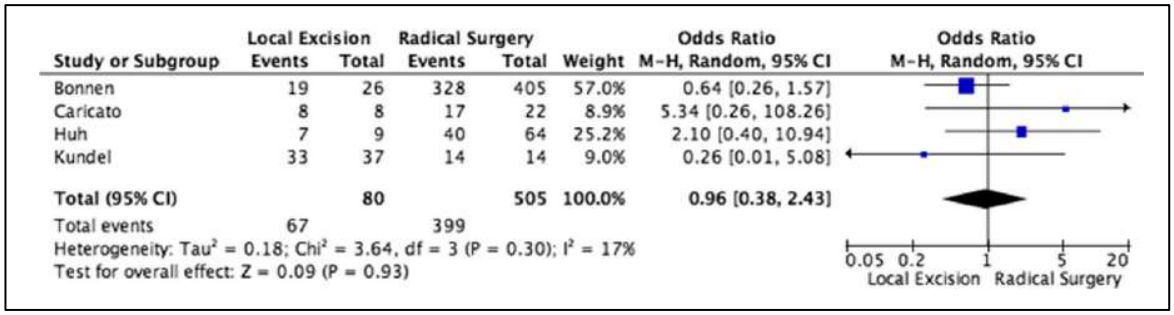

Figure 10 Overall survival across all stages

DISCUSSION

The findings of this systematic review and meta-analysis suggest that there are no differences in LR, DFS and OS rates between RC patients submitted to CRT and LE or RS. Currently, in the majority of patients, radical excision with or without neoadjuvant CRT is still recommended [17]. However, a significant proportion of rectal cancers regress in size following CRT and thereby potentially become amenable for LE. This may offer a theoretically safer alternative to RS, especially amongst the elderly and comorbid patient groups.

The oncological safety and functional outcomes of LE following CRT require consideration:

1. Local Recurrence

The first study reporting on LE following CRT was done by Marks *et al* [355]. This study demonstrated a 21% LR rate, although patient numbers were limited (n=3/14). The study population was composed largely of patients with tumours up to cT2N0 and those who were unfit for radical procedure. In this study, both patients with cT2N0 cancer developed LR. All three patients with LR had grade II mucinous tumours that seem to be associated with aggressive behaviour and higher recurrence rates than non-mucinous cancers [356]. Another series, one of the largest, reported by Guerineri *et al* [357] documented a LR rate of 4% in 175 patients including cT2 and cT3 tumours after 81 months follow-up. Conversely, Parks and colleagues [355] reported no recurrences in a case series including patients staged as cT2–T3 N0. In a review, Smith *et al* [356],[358] observed a LR rate of 0-23% in cT2-T4 rectal cancers treated with CRT followed by LE. Overall, 5-year LR after RS is described as 6–8%.

Although there are single series reporting outcomes after LE for RC undergoing CRT, there are very few that compare this strategy with RS. In our metanalysis, 7 studies presented data on LR for all stages and only 2 presented it for cT3 tumours. Since the oncological outcomes suggested no difference between CRT+LE and CRT+RS, it may be argued that less invasive surgery may represent a viable alternative to RS for disease control. This may be particularly relevant to discussions with patients highly averse to a stoma, as well as those that represent a prohibitive perioperative risk. However, the majority of studies included were based in pre CRT staging and did not detail post-CRT one.

Currently LE after CRT is being mainly offered or thought to be acceptable as a palliative treatment of advanced cancers or in patients not wishing to undergo major surgery that may necessitate stoma formation [357],[350].

2. Survival

Regarding survival, after a mean follow-up of 55 months, Schell and colleagues reported survival of all 11 patients treated with CRT+LE [359]. This case series included advanced cT3 rectal cancers receiving CRT. However, their selection criteria for LE included tumours staged ypT1 after CRT. Over a longer period of follow-up (81 months), Guerrieri *et al* reported an OS of 77% in cT3 tumours and 90% in cT2 tumours [357]. Similar results were reported by Callender *et al* in cT3 tumours (74% 10 year OS) [350]. At the same time, RC treated with CRT and radical excision showed a 5-year OS of 74% [360]. In our systematic review and meta-analysis, 4 studies presented data on survival across all stages and only results reporting at least 10 years of follow-up were considered. There was no statistically significant difference in OS between LE and RS post-CRT (84% versus 79%, respectively)

DFS reflects survival in the absence of local or systemic recurrence. The German Rectal Cancer Study Group reported a DFS rate of 68% after 5 years of follow-up in patients after CRT+RS. Higher rates

of DFS have been reported by Guerrieri *et al* in ypT2 tumours (90%) and ypT3 tumours (77%) after a median 81 months of follow-up in patients having TEM surgery following radiotherapy [357]. In our metanalysis, 4 studies presented data on DFS across all stages and 2 studies in cT3 tumours. Our results did not demonstrate a significant difference in DFS between LE and RS post-CRT over 60 months of follow-up. Similar results were obtained in the subgroup analysis of T3 tumours. The RCT by Lezoche *et al* also showed no difference in DFS [352].

3. Functional outcomes

All studies included in the current analysis were primarily investigating oncological outcomes and not function. Having said this, post-operative bowel function and the requirement of a stoma (either temporary or permanent) are important to patients, as they can significantly impact on quality of live. Importantly, CRT treatment *per se*, in the absence of operative intervention, may adversely affect sphincter function [359][361].

After CRT followed by LE, Marks *et al* reported good defecatory function in 13/14 patients and only 1/14 required a colostomy due to poor sphincter function [362]. Schell *et al* have also reported the impact of low RC treatment on sphincter function [363]. Their findings demonstrated that 2/11 patients suffered sphincter-related morbidity after CRT and LE. One patient underwent successful repair for lax sphincter and one suffered temporary faecal urgency that resolved spontaneously.

However, radical resection is also associated with function disturbances. A study by Do and colleagues reported good to excellent (64%) and fair (36%) sphincter function in patients after low anterior resection (LAR) with preoperative CRT [364]. Their definition of fair sphincter function included four or more bowel movements and moderate faecal soilage with no incontinence. By removing the rectal reservoir and inducing changes in pelvic nerve function, low anterior resection may lead to symptoms of faecal urgency, increased frequency of defecation and faecal soiling. It is conceivable that CRT, which itself can damage the anal canal sphincter's musculature or nerve supply, may potentially compound the effect of TME surgery [352].

Furthermore, in LE techniques, there is also an associated conversion rate to an abdominal procedure, reported in the literature as being 5.7% (n=6/105)[365]. Reasons for such conversions include inaccessibility of lesions, large tumours and breach of the peritoneum. In our metanalysis, peritoneal breaching occurred in 9.5% (n= 10/105). In all but two patients, the peritoneum was closed transanally. Interestingly, there was a late rectal perforation in one patient necessitating APER with permanent colostomy. While rectal perforation appears to have no effect on short- and long-term outcomes, it may prolong operative time and length of hospital stay [350].

Limitations of this metanalysis

We aimed to investigate LR and survival rates after CRT and LE by assessing the studies reporting comparative data. However, this proved difficult because very few studies did a direct comparison between LE and RS post CRT and there was only one RCT reporting these outcomes [352]. Moreover, this RCT included cT2 stage patients. All other studies were observational/ cohort studies that presented only initial staging, not always discriminating results by stage neither considering post CRT re-staging.

Although these studies were assessed (Table 15) for consistency using NICE guidelines (www.nice.org.uk), in the absence of RCTs, it is difficult to interpret the results with accuracy. Overall, this metanalysis only included data from 8 studies, of which 4 assessed only 15 or fewer patients undergoing LE. Median follow-up varied significantly with some studies having nearly 10 years of follow-up. Although there was not a significant statistical heterogeneity, there was noteworthy methodological heterogeneity between the included non-randomized studies.

Table 15 Quality assessment of the selected article

1	Case series collected in more than on centre, i.e. multi-centre study (NICE score) max 8
2	Is the hypothesis/ aim/ objective of the study clearly described?
3	Are the inclusion and exclusion criteria clearly reported?
4	Is there a clear definition of the outcomes reported?
5	Were data collected prospectively?
6	Is there an explicit statement that patients were recruited consecutively?
7	Are the main findings of the study clearly described?
8	Are outcomes stratified (e.g. by disease stage, abnormal test results and patient)
Quality of assessment for case series (adapted from NICE): yes=1, no=0, score_/8	
The combined scores of the above papers used for analysis	
Bannon *et al* [348]	5
Bonnen *et al* [349]	4
Callender *et al* [350]	5
Habr-Gama *et al* [151]	4
Huh *et al* [354]	4
Kundel *et al* [353]	5

The selection criterion for LE was also not standardized. Bonnen and colleagues selected patients for LE if they refused stoma, had significant medical comorbidity or if there was cCR after CRT [349]. Similar reasons were cited by Callender [354],[366]. Based on large series, it appears that for cT3 cancers, 6 to 9% of LE is performed after CRT [367],[368].

Also, there was a substantial variability in many critical technical issues, such as marking/ tattooing the original tumour margins before neoadjuvant therapy, using pre-treatment tumour size or stage as exclusion criteria, stating the lateral excision margins, and even discrepancies in the surgical procedure itself, sometimes showing total mesorectal neglect.

Comparison of LE versus RS after CRT should be made for similar staging and criteria. This could be a potential pitfall when comparing outcomes of these 2 options. In fact, tumours showing regression may

behave in a less aggressive way. The technical aspect of LE may also differ. In this study, the authors performed LE to include local mesorectal excision and 1 cm of normal mucosa, as well as excision of the internal sphincter in lower tumours. As mentioned above, LE can be performed by classic transanal full-thickness excision or minimally invasive techniques. As such, in order to meaningfully compare outcome between these surgical approaches, all these factors need to be taken into account.

More recent studies

As stated above, until recently, the literature concerning LE after CRT in RC was based mainly on retrospective single-institution case series, with variability in selection and inclusion criteria, heterogeneous tumour characteristics and different follow-up. These single series overall showed that LE after CRT is a valid option with good oncological outcomes in patients with complete or very good response (ypT0-1) [350],[378]-[384].

In the last few years, data have also been collected from other multicentre, long-term and randomized trials. Still, very few authors compare LE and RS in post CRT setting. After the publication of our metanalysis, only 4 more studies were published comparing LE and RS post CRT in RC.

Creavin *et al* [373] published the prospective results of 60 of 362 patients with LARC who were treated with an organ-preserving intention after CRT. A surveillance “Watch and Wait” program was offered to patients with a cCR and LE was performed in those who responded but with a residual ulcer less than 3 cm. Fifty patients underwent LE (of whom 15 patients (30%) had to undergo salvage TME) and 10 patients underwent a WW procedure. There was no significant difference in OS (85.6% versus 93.3%, P= 0.414) or DFS (78.3% versus 80%, p=0.846) when the outcomes of radical surgery (302 patients) were compared with organ preservation. Tumour regrowth occurred in 4 out of 45 (8.9%) patients who had organ preservation.

The GRECCAR 2 trial [376], the second prospective randomized trial comparing LE with TME after CRT, demonstrated that in patients with good clinical response after CRT there was no significant difference in 3-year LR and OS between groups (5% and 6% LR and 78% and 76%, respectively). However, this study did not demonstrate any benefit of LE over TME because many patients in the LE group ended up receiving a completion TME that increased morbidity and compromised the potential advantage of LE.

Yang *et al* [375] compared oncological outcomes between LE and TME in ypT2-stage rectal cancer after CRT. With a median follow-up of 57 months, this study showed that, in the LE group, LR occurred more frequently (18 vs. 4%; p = 0.034). Likewise, 5-year LR-free survival (76 vs. 96%; p = 0.006) and overall survival (79 vs. 93%; p = 0.045) were significantly lower in the LE group. In this setting, the high local failure rate and poor oncological outcomes for ypT2-stage patients recommends salvage surgery.

Recently, Calmels *et al* [377] reported a case-matched study comparison of LE in high-risk patients (aged patients with severe comorbidity and/ or indication for APER) versus TME in patients with initial cT3–T4 and/or cN+ low/ mid RC and cCR or near CR after CRT. No significant difference was noted between groups in terms of severe postoperative morbidity, definitive stoma rate and long-term oncological outcome. Furthermore, after LE, the overall morbidity rate was lower and functional results better. This represents a safe alternative to TME in selected patients with complete or near-complete response and with severe comorbidities, indication for APER or difficult intersphincteric resection.

Controversial aspects in LE after CRT

There are still controversial aspects concerning LE performed after CRT, in particular tumour scatter and fragmentation, nodal involvement, completion and salvage surgery and LE morbidity.

1. Scatter cells and fragmentation

After undergoing CRT, patients are restaged with MR, digital and endoscopic examination. However, there is a insufficient correlation between mrTRG and pathological TRG (pTRG) [378]. Moreover, MR accuracy for restaging RC after preoperative CRT is limited [390]-[392] as is clinical evaluation [382], and biopsies for excluding residual tumour have low negative predictive value [383],[384]. This is the main limitation of the "Watch and Wait" approach that relies in the accuracy of the assessment of cCR. For some authors, because cCR does not always coincide with pCR, excision of the scar is required, to confirm the pCR. Likewise, in the presence of a very good but incomplete response there is still doubts of what are residual mucosal abnormalities (RMA) [385],[386]. In this view, LE remains theoretically the best way to identify tumour response to neoadjuvant CRT (ypT) serving also as a decision guide.

The pathological evaluation of the response scar is, however, not a perfect solution since it has been reported that "nests" of viable tumoral cells can be found separated from the main tumour site or mucosal scar, scattered among radiation induced fibrosis underneath normal mucosa, extending for several cms. This "tumour scatter" concept means the possible presence of cancer cells outside the visible ulcer or in the absence of one (Fig 11) [387],[388].

In fact, after finding that 53% of TEM patients had lateral intramural spread (tumour cells apart from the residual cancer in the absence of discontinuity, underneath normal mucosa) extended into the submucosa with a mean distance of 4.8 ± 2.4 mm (maximum 7.2 mm), some authors [389] changed their practice when performing LE, and started to consider margins of at least 15 mm from the visible residual cancer. Furthermore, a recent study has shown that residual tumour cells can be detected mainly in the deepest layers of the rectal wall, thus misleading clinical evaluation [390]. Also, when fibrosis remains after CRT there is still a high number of cancer cells left within fibrosis [391]. This is an argument for the consideration of adjustment of the LE margins. Overall, considering the risk of viable cells distant to the visible alteration, excision of the RMA or scar may not allow excision of the entire residual cancer.

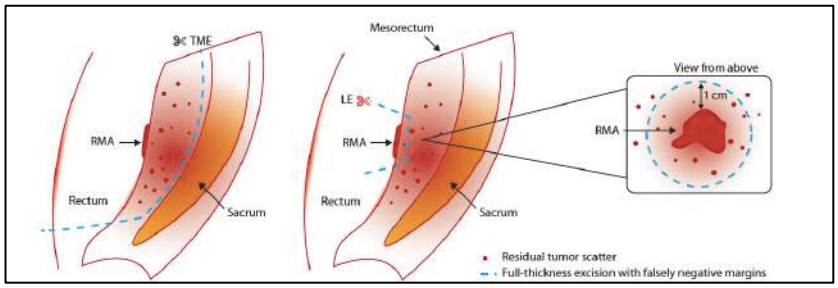

Figure 11 Local excision (LE) of a residual mucosal abnormality (RMA) may have falsely negative margins if significant tumour scatter is present. Reproduced from *Perez et al Local excision techniques for rectal cancer after neoadjuvant chemoradiotherapy: what are we doing? Dis Colon Rectum, 2017 Feb;60(2):228-239*

2. Nodal involvement in LE

The ideal candidate for LE would be the patient with minimal risk of node positivity and tumour restricted to the bowel wall. The question arises: is TME necessary in clinical cN0 tumours localized to the bowel wall? If the tumour is just on the rectal wall, what is the reason for performing a radical surgery?

In RC, LR is associated with N+, tumour invasion, lymphovascular invasion and less histological differentiation. After neoadjuvant CRT, the risk of mesorectal lymph node involvement is correlated with ypT stage. In fact, the ypT classification is a reliable predictor of ypN status. There are authors that report low rates of positive LN in ypT0 tumours [392],[393] unlike others that report higher rates [394]. Overall, nodal involvement is observed in 4–28% of patients with a ypT0/T1 tumour, 16–28% with a ypT2 tumour and over 40% in ypT3. Control of nodal metastasis is a major determinant of LR and even in patients with cCR risk of positive nodes remains.

This why most authors agree that organ preservation with LE can be proposed only for ypT0, ypTis or ypT1 tumours with R0 resection [395]. On the contrary, LE is considered inadequate in yp ≥ T2 even in case of R0 resection. Also, in the presence of an R1 resection or lymphovascular invasion LE is insufficient. In these cases, salvage TME should be proposed. Eventual alternative strategies such as LE extending to the mesorectal fascia to try to overcome this node positive risk can be considered [396],[397].

3. Completion and salvage surgery after failed LE

Radical surgery after LE following CRT is performed either for adverse pathological features (completion surgery) or for local recurrence (salvage surgery).

A contentious issue relates to the completion surgery after failed LE that may not be followed by the same survival rate as initial radical surgery. A recent meta-analysis [396] concluded that completion surgery (LE followed by TME), is associated with higher incomplete mesorectal excision rate (OR 5.74; 95% CI 2.24-14.75; $p \leq 0.0003$) than primary radical surgery (TME alone). Moreover, van Gijn *et al* [397] has reported 10.2% LR in completion TME compared to 5.2% of the primary TME group (HR 6.8; $p < 0.0001$). Moreover, good TME specimens after completion surgery have higher DFS in comparison with poorer specimens [76]. The problem is that it is more difficult to get a good TME specimen after CRT and LE due to the full-thickness excision, residual fibrotic scar and CRT, which can further compromise outcomes [76],[398]. Likewise, patients who undergo completion surgery have a high rate of major complications [398], with a bowel function and quality of life significantly poorer than those who undergo LE or TME alone [399]. Finally, a significant number of patients end-up refusing completion surgery [363],[393].

Another controversial issue relates to salvage surgery. In the presence of LR in patients with previous LE post CRT, one would expect that salvage surgery would allow safe TME and a sphincter saving procedure, if it would have been initially possible. If outcomes of salvage surgery for LR after LE in early RC are good, results for LR following CRT and LE are poor. The main difference is the presence of neoadjuvant

CRT. It seems that LE post CRT compromises the TME planes, causes fibrosis and high R1 resections, and subsequent radical surgery have suboptimal TME specimens and higher anastomotic leaks. In a study by Perez *et al* [346], among patients who underwent salvage surgery there was a 50% 2-year LR (re-recurrence), inferior specimen quality (87.5% CRM positivity) and 87,5% APER rate. In addition, LR, especially in wound breakdown cavities, is notoriously difficult to manage, and salvage surgery in this context is associated with high R1 resection and subsequent re-recurrence. It is for these reasons that some surgeons have doubts as to whether full-thickness excision after neoadjuvant CRT is an acceptable policy in the management of RC especially in patients who are otherwise fit for radical surgery [352].

4. Morbidity of LE after CRT

LE for RC is not without complications and a learning curve is associated with the technique [400]. In a systematic review of LE after CRT, pooled incidence of morbidity was as high as 23.2% (95% CI, 15.7%–31.7%), with 9.9% suture-line dehiscence and 10.7% rectal pain, the 2 most relevant complications [401]. Other complications included rectal bleeding, perirectal sepsis and intraoperative breach of the abdominal cavity. Haemorrhage, pelvic peritonitis with need for re-intervention or defunctioning ileostomy have also been described.

In the study by Lezoche and colleagues, there was a 13% (n=5/38) rectal dehiscence, requiring parenteral nutrition and antibiotics [402]. Another single surgeon series of TEM procedures performed for both rectal adenomas and adenocarcinomas reported 13% (33/262) morbidity, including pelvic sepsis (2.7%), bleeding (2.7%), rectal stenosis (1.5%) and a mortality risk of 0.8%. Lesions resected within 2 cm of dentate line were associated with a significantly higher risk of pelvic sepsis, presumably due to lack of mesorectum in this region [403].

A key point seems to be related to neoadjuvant CRT, since it has been demonstrated that early postoperative morbidity, wound dehiscence and readmission rates are more significant in patients undergoing TEM following CRT than TEM alone [401],[402]. There are 30% hospital readmissions and 13.7% of re-interventions, namely for constructing a loop stoma, re-suturing a dehiscent suture line but also for APER or trans-sacral debridement due to permanent sepsis or sinus [402].

Complications of LE also include rectal stenosis and poor sphincter function. One study investigating sphincter function after TEM using anorectal physiology techniques, found that in the early post-operative period there was a loss of the recto-anal inhibitory reflex, reduction in rectal maximum tolerated volumes and increase frequency of bowel motions. The authors reported patients experiencing episodes of temporary urge incontinence, possibly due to damage to the internal anal sphincter [403],[404].

CONCLUSION

The original paper in this chapter, a systematic review and meta-analysis of the current published literature, suggests that there is no statistical difference in LR, OS and DFS in RC patients (across all stages and in cT3 specifically) undergoing CRT and LE versus CRT plus RS. While the results of our metanalysis should be interpreted with caution because the majority of studies included were observational and selection criteria may have varied, they are in agreement with 4 other more recent prospective and multicentric publications.

This seems contrary to the notion that LE is inadequate in yp ≥ T2, in R1 resection, in the presence of LVI or EMVI, but its not. What might explain our metanalysis results is the fact that the determinant of LR and survival is not the baseline clinical staging but the post CRT staging that reflects the tumour's biologic behaviour. In fact, in our review, some studies compared patients addressing baseline staging and not post CRT restaging. The determinant point is, in fact, the post neoadjuvant therapy stage and not the initial one.

Nevertheless, this meta-analysis opens debate regarding the requirement for radical rectal excision for low RC especially in patients with a good response to CRT. The fact is that LE following neoadjuvant therapy ultimately depends on the effectiveness of CRT: if CRT is very effective obtaining a complete response (ycT0), positive nodes and LR rates are low. If there is an incomplete response, these rates become higher. So, if lymph nodes are positive after CRT, patients are optimally treated by radical excision with regional lymph node clearance [405]. However, if there is significant tumour regression and ycN0 status, LE is a potential alternative that, in this limited statistical analysis of the available literature, appears to offer comparable oncological outcome. This may be of particular importance in the elderly and comorbid patient for whom radical surgery harbours significant morbidity and mortality risk. Nonetheless, one should bear in mind issues such as tumour scatter, the morbidity of LE after CRT and the problematic of salvage surgery in this setting.

In summary, in the presence of a cCR (ycT0), because post CRT LE has wound healing problems and surveillance is facilitated by the absence of scar, observation alone can be proposed. In patients with an incomplete response contained in the mucosa or submucosa (ycT1N0), with significant comorbidity or refusing APER, LE could be offered, although TME remains the gold standard treatment. An immediate completion TME is required in case of unfavourable pathology.

Original Paper 7

Loop ileostomy in Rectal Cancer Surgery: Factors Predicting Reversal and Stoma Related Morbidity

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ABSTRACT

***Background**: Loop ileostomy is performed in rectal cancer surgery to decrease the impact of anastomotic leak but it is associated with a significant complication rate.*

***Objectives:** This study aimed to analyse the morbidity related to diverting ileostomy and to identify factors predictive of complications related to stoma management and reversal, as well as conversion into a permanent one.*

***Methods**: A retrospective study was conducted on 112 patients submitted to oncological rectal resection and defunctioning ileostomy in a Portuguese colorectal unit between March 2012 and March 2019.*

***Results:** Loop ileostomy was responsible for 13% of index surgery morbidity and 15% of patients readmissions due to high output, stoma stenosis and parastomal hernia. Ileostomy was reversed in 89% cases with 7% Clavien-Dindo ≥ IIIb complications. An association was established between diabetes and higher stoma management morbidity (OR: 3.28 [95% CI: 1.039-10.426], p = 0.041). Likewise, diabetes (OR: 0.17 [95% CI: 0.038; 6.90], p=0.015), oncological disease stage ≥ III (OR 0.10 [95% CI: 0.005; 0.656], p=0.047) and index rectal surgery morbidity (OR 0.23 [95% CI: 0.052; 0.955], p=0.041) were associated with less ileostomy closure. Complications of the index surgery also related to higher stoma reversal morbidity (OR 5.11 [95% CI: 1.665; 16.346], p=0.005).*

***Conclusions:** Diabetes and complications of index rectal surgery were identified as predictive of ileostomy morbidity, closure rate and associated complications. It is essential to adjust treatment decisions to patient's morbidity risk and adopt a more selective approach concerning the use of a derivative ileostomy in rectal cancer surgery.*

Keywords: rectal cancer, derivative ileostomy, morbidity, prognostic factors

INTRODUCTION

Rectal cancer (RC) represents more than one third of all colorectal neoplasia and TME is the gold standard treatment for mid-lower rectal tumours [59]. TME adoption has contributed to a reduction of local recurrence, however the incidence rates of postoperative surgical morbidity remained almost unchanged. Anastomotic leak, reported up to 23%, is the most feared complication [11],[406],[407]. In order to mitigate systemic response related to anastomotic leak, there is a general trend to perform a diverting stoma in distal anastomosis and in patients who received neoadjuvant radiotherapy [12]. However, this protective effect needs to be balanced against stoma morbidity [13].

Loop ileostomy complication rate is as high as 35% and can include skin problems, leakage from the stoma appliance, high output syndrome, parastomal hernia or prolapse [408], [409]. Equally, stoma reversal has an overall complication rate up to 20%, postoperative ileus and surgical site infection (SSI) being the most common [410],[411]. Moreover, approximately 28% of defunctioning stomas become permanent, mostly due to oncological disease progression or need for adjuvant chemotherapy[412]. Another important matter of debate is timing of stoma reversal and, accordingly to the literature, early closure does not seem to be associated with higher postoperative complications [410], [411].

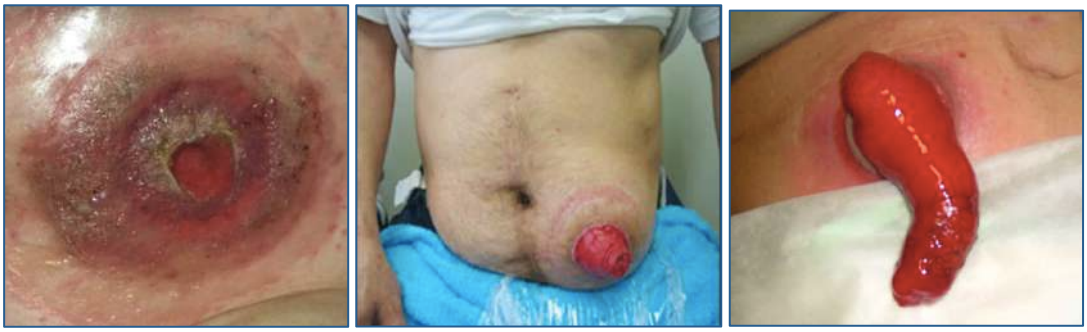

Figure 12 Ileostomy related complications. Skin irritation, parastomal hernia and prolapse.

RATIONAL AND AIMS

Rational: Loop ileostomy is performed in rectal surgery to decrease morbidity and mortality associated with dehiscence of colorectal anastomosis. Although ileostomy does reduce the consequences of a leak, the majority of patients does not have this problem and are unnecessarily exposed to stoma potential morbidity. This study hypothesized that preoperative factors can predict ileostomy related morbidity, probability of ileostomy closure and associated morbidity, allowing individualized decisions on endorsing or avoiding diversion.

Aims: The aim of this study was to analyse stoma closure and morbidity associated with ileostomy performed in RC and identify risk factors predictive of morbidity and stoma closure as well as transformation into a permanent stoma. The work also wanted to investigate whether surgical techniques play a role, in a context of disagreement about the optimal anastomotic procedures.

MATERIALS AND METHODS

Study design

This was a retrospective study of all patients submitted to radical rectal resections for cancer between March 2012 and March 2019 in Hospital Beatriz Ângelo in Lisbon, followed until October 2020. Data were gathered from the electronic hospital prospective database.

Eligibility and perioperative management

Patients with adenocarcinoma of the rectum (stages I to IV, AJCC TNM 8th ed., 2017), aged over 18, submitted to TME with defunctioning loop ileostomy were eligible. Patients synchronously submitted to other oncological resections were also included. Patients with resections without diverting stoma, end-colostomy or abdominoperineal resection were excluded.

Criteria for constructing a diverting loop ileostomy in the context or rectal cancer surgery were performing anterior resection with TME or partial mesorectal excision with extraperitoneal anastomosis, pouch surgery or neoadjuvant radiotherapy.

From October 2017 onwards patients were treated according to the Enhanced Recovery After Surgery (ERAS) protocol, systematically introduced in our institution for colorectal surgery.

High output stoma was defined as the one producing more than 1L effluent/ day. Stoma was prophylactically addressed with adapted antidiarrheal diet, with electrolyte mix and loperamide. The ileostomy reversal procedure was scheduled before adjuvant chemotherapy (approximately 21 days after index procedure) or after its completion. Prior to stoma reversal, the colorectal anastomosis was evaluated with digital examination, rectosigmoidoscopy and gastrografin enema.

Morbidity and mortality

Morbidity related to loop ileostomy was divided in: 1) morbidity of index surgery caused by ileostomy (during the first 30 post-operative days or during the admission for the rectal surgery), 2) morbidity associated with ileostomy management (after discharge from the index surgery admission) and 3) morbidity associated with ileostomy closure (during the first 30 post-operative days or during admission for stoma reversal).

The former 2 included dehydration due to high output, parastomal hernia, ileostomy stenosis, peri-ileostomy abscess or bleeding and hospital admissions or further surgeries resulting from stoma complications. Morbidity associated with stoma closure was categorized according to the Clavien-Dindo classification [317] and comprised SSI, anastomotic leak, ileus (absence of bowel function on postoperative day 5), gastrointestinal bleeding or small bowel obstruction. Skin problems and leakage from the stoma appliance were not included in this analysis.

Regarding the index rectal surgery, colorectal anastomotic leak was defined according to the Rectal Cancer Study Group including clinical, radiological leak and perianastomotic, pelvic abscess or recto-vaginal fistula [316].

Endpoints

Primary endpoints were rate of stoma reversal, morbidity related to loop ileostomy management and to stoma closure. Secondary endpoints were clinical factors predictive of ileostomy morbidity, of complications associated with the reversal and of its transformation into a permanent stoma. Finally, the impact of time till closure and anastomotic techniques on morbidity were also evaluated.

Statistical analysis

Survival analysis was performed through Kaplan–Meier (KM) statistics. Logistic regressions were used to correlate each variable with the outcomes defined: ileostomy complications, ileostomy closure and post closure complications. Only variables with $p \leq 0.20$ in univariate logistic regression or considered clinically relevant were selected to multivariable logistic regression. Significance level was set at 0.05.

Fisher's exact test and ANOVA's test were used to test the association between intersurgical time-closure morbidity, respectively. No logistic regression analysis had evidence of poor fit (Hosmer and Lemeshow Godness of Fit). Data was analysed with R (version 4.0.2, 2020-06-22, "Taking Off Again").

RESULTS

Patient clinical parameters

During the study period, a total of 220 consecutive RC patients were submitted to surgical treatment of which 112 were included in this analysis (Fig 13). Demographic and clinical parameters are summarized in Table 16.

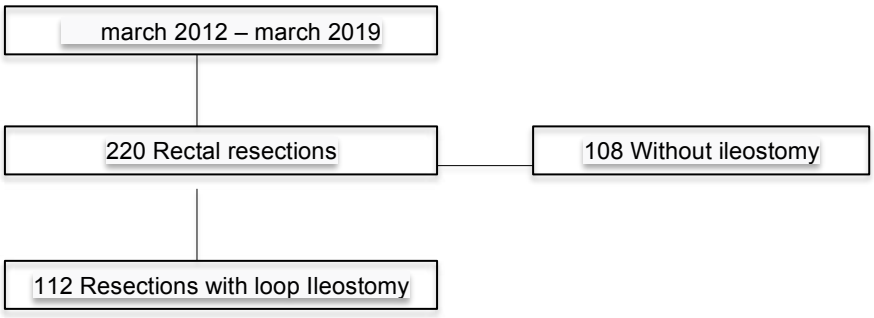

Figure 13 Volume of radical resections for rectal cancer. Unit's volume of radical resections (non exenterative) for RC during the elected period of the study.

Table 16 Patient clinical parameters

Clinical parameters		Patients (n = 112)
Gender, n (%)	Female	38 (33.9)
	Male	74 (66.1)
Age, median		67 (60-74)
Obesity, n (%)	No	99 (88.4%)
	Yes	13 (11.6%)
Respiratory comorbidities, n (%)	No	98 (87.5%)
	Yes	14 (12.5%)
Cardiac comorbidities, n (%)	No	81 (72.3%)
	Yes	31 (27.7%)
HBP, n (%)	No	48 (42.9%)
	Yes	64 (57.1%)
Diabetes, n (%)	No	85 (75.9%)
	Yes	27 (24.1%)
ASA score, n (%)	I	1 (0.9%)
	II	69 (61.6%)
	III	40 (35.7%)
	IV	2 (1.8%)

Obesity: Body Mass Index over 30; Cardiac comorbidities: disrritmias (atrial fibrillation, need of pacemaker, left or right cardiac blockage), coronary disease, cardiac insufficiency, aortic or mitral stenosis or insufficiency and past medical history of stroke or myocardial infarction; Respiratory comorbidities: chronic obstructive pulmonary disease (COPD), sleep apnoea or chronic pulmonary embolism; HBP high blood pressure, ASA American Society of Anaesthesiologists. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3).

Staging, neoadjuvant therapy and index surgery

Of the 112 patients, 111 (99%) were treated for RC and one (1%) for endoscopically non-resectable adenoma with high-grade dysplasia (Table 2). At diagnosis, 66 (60%) patients presented disease stage III with a median CEA of 1.7 ng/mL (0.8–3.2). 81 (72%) patients had some type of neoadjuvant treatment while 31 (28%) underwent direct resection. Twenty-one (19%) patients underwent a TaTME and 91 (81%) an anterior resection (AR), 10 with synchronous liver metastasectomy or colectomy. Laparoscopic approach was used in 73 (65%) patients with 12 (11%) cases requiring conversion.

There were 53 (47%) postoperative complications of the rectal surgery, 22 (20%) Clavien-Dindo≥ IIIb. Overall anastomotic insufficiency rate was 11% (12/112), related to 3 peri-anastomotic collections treated with antibiotic (Clavien II), 1 leak treated with endo-SPONGE® (Clavien IIIa) and 8 that needed surgical re-exploration (Clavien IIIb).

Seventy-two (64%) patients underwent adjuvant chemotherapy, 67 (93%) before ileostomy reversal (Table 17).

Table 17 Staging, neoadjuvant therapy and index surgery

Clinical parameters		Patients (n = 112)
CEA (ng/mL)		1.7 (0.8-3.2)
Stage	cTxN0M0	1 (0.9%)
	I	24 (21.6%)
	II	8 (7.2%)
	III	66 (59.5%)
	IV	12 (10.8%)
	NA	1 (0.9%)
Tumour location in rectum, n (%)	Superior 1/3	36 (32.1%)
	Middle 1/3	37 (33.0%)
	Inferior 1/3	39 (34.8%)
Neoadjuvant treatment, n (%)	CT	3 (2.7%)
	LCCRT	73 (65.2%)
	SCRT	5 (4.5%)
	Surgery upfront	31 (27.7%)
Surgical procedure, n (%)	AR	91 (81.3%)
	TaTME	21 (18.7%)
Surgical approach, n (%)	Laparoscopy	61 (54.5%)
	Laparotomy	39 (34.8%)
	Conversion	12 (10.7%)
Morbidity Index surgery, n (%)	No	59 (52.7%)
	Grade I	8 (7.1%)
	Grade II	18 (16.1%)
	Grade IIIa	5 (4.5%)
	Grade IIIb	20 (17.9%)
	Grade V	2 (1.8%)
Adjuvant CT, n (%)	No	40 (35.7%)
	Prior to stoma closure	67 (59.8%)
	Post stoma closure	5 (4.5%)

CEA carcinoembryonic antigen, Stage American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017, NA not applicable, CT chemotherapy, LCCRT long course chemoradiotherapy, SCRT short course radiotherapy, AR anterior resection, TaTME transanal total mesorectal excision. Morbidity according to Clavien-Dindo [317]. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3).

Ileostomy reversal

From the initial 112 patients, 2 died in the index surgery post-operative period, 1 with the ileostomy closed and the other without. So, from the 111 patients eligible for closure, 99 (89%) underwent ileostomy reversal with a median time interval from primary procedure of 8.4 (5.9-11.9) months. The majority (60%) of patients had a stapled side-to-side anastomosis. Median duration of reversal procedure was 80 (60-100) minutes, skin closure technique was a purse-string in 41 (45%) patients and primary closure in 36 (40%)(Table 18).

Loop ileostomy as permanent stoma

Twelve (11%) patients did not undergo reversal of the stoma, of which six presented with disease stage III and five with stage IV at diagnosis (Table 18). Reasons for not closing the stoma were disease progression in six patients, two colorectal anastomotic strictures, one metachronous second cancer, two patient refusals and one colorectal anastomotic leak

Table 18 Ileostomy reversal

Clinical parameters		Patients (n = 111)
Ileostomy reversal, n (%)	No	12 (10.8%)
	Yes	99 (89.2%)
Time till ileostomy reversal, months		8.4 (5.9-11.9)
Pre op total seric protein, median		6.7 (6.2-7.2)
Pre op seric albumin, median		4.3 (3.8-4.4)
Pre op CRP, mg/dL, median		0.71 (0.2-1.2)
Ileostomy closure time, min		80 (60-100)
Surgical anastomosis, n (%)	Side-to-side handsewn	4 (4.1%)
	Side to side mechanical	58 (59.8%)
	End to end manual	35 (36.1%)
	NA or ND	14
Surgical skin closure, n (%)	Purse-string	41 (45.1%)
	Primary closure	36 (39.6%)
	Primary closure over drain	14 (15.4%)
	NA or ND	20
Time till diet tolerance, days (median)		2.5 (1-7)
Time till bowel transit, days (median)		3 (2-7)
LOS, days (median)		5 (4-8)

One patient that died after index surgery was not eligible for ileostomy closure rate and was not included in this analysis. CRP seric C reactive protein, LOS length of hospital stay, NA not applicable, ND not discriminated. Continuous variables are reported as median, range of first and third quartiles (Q1-Q3)

Ileostomy related morbidity

Loop ileostomy was responsible 13% of morbidity cases of the index RC surgery, namely one high output stoma, one peristomal bleeding, two peristomal abscesses, one ileostomy stenosis with obstruction and two strangulated parastomal hernias that prompted urgent surgical exploration (Table 19).

After discharge from the index surgery, of the 110 patients with loop ileostomies 16 (15%) presented with complications that required 20 hospital readmissions: 18 cases of dehydration due to high output, one obstruction secondary to ileostomy stenosis and one strangulated parastomal hernia. During follow-up, six (5%) patients also developed paraileostomy hernias but these did not warrant surgical intervention or readmission (Table 19).

Likewise, 24 (24%) patients had complications of ileostomy closure, four (4%) of which were Clavien-Dindo IIIb: one anastomotic leak, one small bowel obstruction, one iatrogenic enterotomy and one small bowel ischemia. There were three (3%) deaths in the postoperative period, 2 due to anastomotic leak and one to pneumonia. Reoperation rate was 6.1 (Table 19).

Table 19 Ileostomy associated morbidity

Clinical parameters		
Morbidity of index surgery caused by ileostomy, n (%)		7/ 112 (6.3)
Clavien-Dindo, n (%)	Grade II	5 (4.5)
	Grade III b	2 (1.8)
Type	High output, dehydration	1
	Ileostomy stenosis	1
	Parastomal hernia	2
	Peri-ileostomy bleeding	1
	Peri-ileostomy abscess	2
Morbidity of ileostomy management, n (%)		22/ 110 (20)
Type	High output, dehydration	14
	Ileostomy stenosis	1
	Parastomal hernia	7
Readmissions		20 (18.2)
Morbidity of ileostomy closure		24/ 99 (24)
Clavien-Dindo, n (%)	Grade I	3 (3.0)
	Grade II	12 (12.0)
	Grade IIIa	2 (2.0)
	Grade IIIb	4 (4.0)
	Grade V	3 (3.0)
Type	SSI	10
	Anastomotic leak	3
	UTI	2
	Ileus	2
	SBO	2
	Gastrointestinal bleeding	1
	Iatrogenic enterotomy	1
	Pseudomembranous colitis	1
	Pneumonia	1
	Fever	1
Reoperation, n (%)		6 (6.1)

Two patients that died after index surgery were not included in the morbidity of ileostomy management data; Morbidity of ileostomy closure included data on the 99 patients that closed their stoma; Morbidity according to Clavien-Dindo classification [317].; SSI surgical site infection, UTI urinary tract infection, SBO small bowel obstruction. Continuous variables are reported as median, range of first and third quartiles (Q1- Q3)

Factors predictive of ileostomy morbidity

In order to test the association between clinical factors and ileostomy morbidity, logistic regression analysis was performed. On univariate analysis, age (OR 1.07 [95% CI: 1.009; -1.149], p = 0.034), diabetes (OR 4.05 [95% CI: 1.335; -12.428], p = 0.013) and high blood pressure (OR 3.82 [95% CI: 1.144; -17.452], p = 0.046) were associated with the presence of more ileostomy complications. In multivariable analysis this association was again demonstrated for diabetes with odds of ileostomy complications 3.28 times greater in individuals with diabetes when compared with patients without (OR 3.28 [95% CI: 1.039-10.426]. p = 0.041) (Table 20). There was no association between stoma morbidity and gender, BMI, respiratory or cardiac co-morbidities, ASA grade, pre-treatment CEA level, stage, neoadjuvant treatment, tumour location, index surgical approach and related morbidity (Table 20)

Table 20 Factors predictive of ileostomy morbidity

Variables	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Female	0.83 (0.283; 2.637)	0.745		
Age	1.07 (1.009; 1.149)	0.034	1.07 (1.004; 1.612)	0.050
Obesity	1.10 (0.160; 4.705)	0.904		
Respiratory comorbidities	1.78 (0.368; 6.676)	0.419		
Cardiac comorbidities	1.70 (0.533; 5.088)	0.347		
Diabetes	4.05 (1.335; 12.428)	0.013	3.28 (1.039; 10.426)	0.041
HBP	3.82 (1.144; 17.452)	0.046		
ASA				
I+II				
III+IV	2.45 (0.841; 7.441)	0.101		
CEA (pre treatment)	0.982 (0.873; 1.005)	0.589		
Stage (AJCC)				
I+II				
III+IV	0.92 (0.304; 3.139)	0.886		
Neoadjuvant therapy				
Direct surgery	0.56 (0.122; 1.904)	0.394	0.40 (0.087; 1.453)	0.196
CT/LCCRT/SCRT				
Tumour location				
High rectum				
Mid rectum	0.17 (0.025; 0.733)	0.183		
Low rectum	0.44 (0.123; 1.431)			
Index surgical procedure				
AR				
TaTME	1.00 (0.285;4.686)	0.999		
Index surgical approach				
Laparotomy				
Laparoscopy	2.02 (0.681; 6.830)	0.221		
Index surgery morbidity	1.52 (0.524; 4.570)	0.442		
Index surgery morbidity				
Clavien-Dindo 0-IIIa				
Clavien- Dindo IIIb - V	0.94 (0.200; 3.268)	0.923		

Factors predictive of ileostomy closure

Overall, 99 (88%) patients had their ileostomy closed. In order to study the influence of clinical factors on ileostomy closure, logistic regression analysis was again performed. Multivariable analysis confirmed a statistically significant association of stoma closure with gender (OR 0.11 [95% CI: 0.005; 0.673], p=0.049), diabetes (OR 0.17 [95% CI: 0.038; 6.90], p=0.015), stage (OR 0.10 [95% CI: 0.005; 0.656], p=0.047) and morbidity of the index rectal surgery (OR 0.23 [95% CI: 0.052; 0.955], p=0.041). Overall, ileostomy closure was less likely in females, patients with diabetes, higher clinical stages and those with complication of the index rectal surgery. No impact on ileostomy closure derived from age, BMI, cardiac comorbidities, high blood pressure (HBP), ASA grade, pre-operative CEA level, tumour location, surgical procedure, adjuvant chemotherapy or morbidity of the ileostomy itself (Table 21).

Table 21 Factors predictive of ileostomy closure

Variables	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Female	0.14 (0.008; 0.753)	0.064	0.11 (0.005;0.673)	0.049
Age	1.01 (0.956; 1.072)	0.647		
Obesity	1.66 (0.285; 31.491)	0.642		
Cardiac comorbidities	2.28 (0.566; 15.317)	0.303		
Diabetes	0.31 (0.094; 1.068)	0.057	0.17 (0.038;0.690)	0.015
HBP	1.16 (0.351; 3.750)	0.798		
ASA				
I+II				
III+IV	0.95 (0.296;3.360)	0.939		
CEA (pre treatment)	1.00 (NA; 1.00)	0.319		
Stage (AJCC)				
I+II				
III+IV	0.17 (0.009;0.931)	0.098	0.10 (0.005; 0.656)	0.047
Tumour location				
High rectum				
Mid rectum	0.47 (0.093; 1.943)	0.314		
Low rectum	0.80 (0.147;3.869)	0.775		
Surgical procedure				
AR				
TaTME	0.77 (0.112;3.167)	0.741		
Surgical approach				
Laparotomy				
Laparoscopy	0.72 (0.205;2.312)	0.587		
Index surgery morbidity	0.52 (0.148;1.672)	0.281		
Index surgery morbidity				
Clavien-Dindo 0-IIIa				
Clavien- Dindo IIIb-V	0.33 (0.098;1.210)	0.079	0.23 (0.052;0.955)	0.041
Adjuvant CT	0.91 (0.213;6.254)	0.904		
Ileostomy morbidity	1.51 (0.454;4.915)	0.488		
Number of readmissions for ileostomy morbidity	0.17 (0.009;1.594)	0.139		

Multiple logistic regression analysis using ileostomy closure as dependent variable. OR odds ratio, CI confidence interval, HBP high blood pressure, ASA American Society of Anaesthesiologists, CEA carcinoembryonic antigen, AJCC American Joint Committee on Cancer TNM Staging Classification for Rectal Cancer 8th ed., 2017, AR anterior resection, TaTME transanal total mesorectal excision. Hosmer and Lemeshow Godness of Fit (p=0.992) indicate no evidence of poor fit.

Factors predictive of ileostomy closure morbidity

On univariate analysis, morbidity of the initial rectal surgery (OR 4.64 [95% CI: 1.550; 14.287], p = 0.006) and adjuvant chemotherapy administered prior to ileostomy closure (OR 7.50 [95% CI: 1.111; 62.875], p = 0.039) were associated with more closure complications (Table 22). In multivariable analysis, only complications of the index surgery were significantly associated with morbidity of stoma closure (OR 5.11 [95% CI: 1.665; 16.346], p=0.005), with patients with Clavien-Dindo equal to or over IIIb having increased odds of complications.

Likewise, although with no statistical significance, there was a trend for an association of diabetes with ileostomy closure morbidity (OR: 2.57 [95% CI: 0.846-7.693], p=0.09). There was no association with age, gender, BMI, respiratory or cardiac co-morbidities, ASA grade, pre-treatment CEA level, clinical stage, neoadjuvant treatment or surgical approach. Also, no impact on complications derived from pre ileostomy closure levels of seric total proteins, albumin, C reactive protein (CRP), presence of a parastomal hernia, adjuvant chemotherapy, different anastomotic and skin closure techniques, morbidity of the ileostomy management and number of readmission derived from it. Finally there was no impact of increase time to ileostomy closure on complications (Table 22).

Table 22 Factors predictive of ileostomy closure morbidity

Variables	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Female	0.69 (0.274; 1.758)	0.429		
Age	1.00 (0.955; 1.047)	0.970		
Obesity	0.24 (0.013; 1.329)	0.181		
Respiratory comorbidities	1.22 (0.309; 4.080)	0.758		
Cardiac comorbidities	1.19 (0.429; 3.113)	0.731		
Diabetes	2.21 (0.766; 6.165)	0.132	2.57 (0.846; 7.693)	0.090
HBP	0.48 (0.187; 1.193)	0.116		
ASA I, II	0.92 (0.349; 2.340)	0.870		
Pre treatment CEA	1.03 (1.002; 1.079)	0.107		
Stage (AJCC)				
III+IV	0.59 (0.229; 1.562)	0.281		
Neoadjuvant therapy				
Direct surgery	1.33 (0.497; 3.426)	0.560		
CT/LCCRT/SCRT				
Surgical procedure				
AR				
TaTME	0.93 (0.312;3.177)	0.905		
Surgical approach				
Laparotomy				
Laparoscopy	0.92 (0.370;2.305)	0.859		
Index surgery morbidity	1.77 (0.710;4.487)	0.223		
Clavien-Dindo 0-IIIa				
Clavien- Dindo IIIb - V	4.64 (1.550;14.287)	0.006	5.11 (1.665; 16.346)	0.005
Adjuvant CT	0.46 (0.179;1.167)	0.100		
Adjuvant CT prior closure	7.50 (1.111;62.875)	0.039		
Ileostomy morbidity	1.22 (0.309;4.080)	0.758		
Number of readmissions for ileostomy morbidity	1.33 (0.051;20.212)	0.837		
Pre op total seric protein	1.19 (0.550;2.648)	0.658		
Pre op seric albumin	0.84 (0.246;2.976)	0.771		
Pre op seric CRP	1.08 (0.907;1.308	0.389		
Ileostomy closure time	1.01 (1.002;1.025)	0.026		

Anastomosis		
S-S handsewn		
S-S mechanical	0.95 (0.112; 20.148)	0.969
E-E handsewn	1.20 (0.134; 25.870)	0.881
Skin closure		
Purse-string		
Primary closure	1.05 (0.379;2.879)	0.926
Time to diet tolerance	1.01 (0.864;1.174)	0.913
Time to bowel transit	1.01 (0.843;1.203)	0.928
Time to stoma closure	1.02 (0.941;1.109)	0.587

Multiple logistic regression analysis using ileostomy closure as dependent variable. OR odds ratio, CI confidence interval, HBP high blood pressure, ASA American Society of Anaesthesiologists, CEA carcinoembryonic antigen, AJCC American Joint Committee on Cancer TNM Staging Classification for Rectal Cancer 8th ed., 2017, CT chemotherapy, LCCRT long course chemoradiotherapy, SCRT short course chemoradiotherapy, AR anterior resection, TaTME transanal total mesorectal excision; CRP C reactive protein, S-S side-to-side anastomosis, E-E end-to-end anastomosis. Hosmer and Lemeshow Godness of Fit (p=0.992) indicate no evidence of poor fit.

DISCUSSION

Loop ileostomy is performed in rectal surgery to decrease morbidity and mortality associated with dehiscence of colorectal anastomosis [413]. The decision to create this defunctioning stoma is influenced by anastomosis site and pre-/intraoperative risk factors for leak [406], [414], [415]. If some procedures have a dehiscence risk that warrants routine diversion (11% for ultralow/ coloanal and 13% for ileal pouch anal anastomosis), others have variable leak rates that question constructing a defunctioning stoma (3-23% for anterior resection) [412],[416],[417]. Overall, leak rate is reported from 5-23% and is associated with considerable morbidity, mortality, higher cancer recurrence, diminished bowel function and quality of life [424]-[426].

Although ileostomy does reduce these poor consequences of a dehiscence, 85-90% of patients do not endure this problem, do not benefit from a stoma and are unnecessarily exposed to its potential morbidity. Proponents of a diverting ileostomy claim a minor negative impact derived from the stoma [421] but arguments for omitting it rely precisely on avoiding associated morbidity, evading intestinal atrophy with immediate use of anal sphincter and the need for only a single hospital admission.

The ileostomy morbidity relates not just to the reversal procedure, often considered a “minor” procedure, but also to the management of the stoma itself. In fact, overall morbidity is reported as high as 35% [408],[422],[423] with skin irritation, retraction, prolapse, dehydration and electrolyte disturbance from high output that often lead to hospital readmissions [436]-[438]. Also, wound infection is reported as high as 18.3%, small bowel obstruction as 15%, enterocutaneous fistula in 0.5-7%, anastomotic dehiscence up to 8% and parastomal hernia up to 12% [418]. Subsequent laparotomy can be needed (3.7%) to close the stoma in the presence of adhesions, obstruction or hernia [420]. Moreover, having an ileostomy significantly impacts on the quality of life [420],[427] and a meaningful proportion of the so-called “transient” stomas are never reversed [406],[421]. Finally, one always has to consider a mortality risk [418].

Currently we still lack precise data on rates of major morbidity associated with ileostomy management and reversal. Likewise, identification of risk factors for these complications could improve patient selection allowing individualised decisions on endorsing or avoiding diversion. In such a controversial setting, this study aimed to evaluate, in a colorectal unit, the stoma closure and morbidity rates

associated with ileostomy performed in RC surgery. We intended to analyse modifiable and non-modifiable risk factors predictive of morbidity and stoma closure and investigate whether surgical techniques play a role, in a context of disagreement about the optimal anastomotic procedure.

In the present study, we observed 22 (20%) cases of complications of ileostomy maintenance, with 16 (15%) patients needing readmission, mainly due to dehydration for high output stoma. Regarding risk factors for this morbidity, univariate analysis identified diabetes, age and high blood pressure as associated with complications. Multivariable analysis, however, validated this association only for diabetes, with odds of complications 3,28 times greater in individuals with this disease when compared with patients without (OR 3.28 [95% CI: 1.039-10.426]. p = 0.041).

We report 89% ileostomy closure rate with a median time to reversal of 8.4 (5.9-11.9) months, higher than some series [420],[427]. This relates to the fact that, in our institution, adjuvant ChT is mainly performed prior to stoma closure, delaying the procedure. In multivariable analysis, there was a statistically significant association between stoma closure and diabetes (OR 0.17 [95% CI: 0.038; 6.90], p=0.015), stage (OR 0.10 [95% CI: 0.005; 0.656], p=0.047) and morbidity of the index surgery (OR: 0.23 [95% CI: 0.052; 0.955], p=0.041). Overall, ileostomy closure was decreased in patients with diabetes, higher clinical stages and complications of the index rectal surgery. It is interesting to note that, in our series, 12 (11%) patients did not undergo stoma reversal mainly related to oncological disease progression. Although treated with a curative intent, these patients had locally advanced and metastatic disease (III and IV) that seem to negatively influence closure.

Our results are in concordance with previous reports that show that an important part of the pretended “temporary” stomas are not closed [421],[428]. In this setting, one might question if, in patients with more advanced disease, the option should be for a non-restorative procedure from the outset. Late colorectal anastomotic strictures or leak, metachronous second cancer and patient refusal were also reasons for non-closing. Interestingly, although delaying reversal, adjuvant ChT did not impact on the rate of stoma closure.

Concerning ileostomy closure morbidity, we observed 24% of complications, 7% equal to or over Clavien Dindo IIIb with 6% re-operation rate. Other groups reported similar results including a meta-analysis that reviewed 6107 patients in 48 studies that showed 17.3% ileostomy closure complications and 3.7% reoperation rate [429]–[433]. In our study, a significant association was identified between complications of the index rectal surgery and complications of ileostomy closure (OR 5.11 [95% CI: 1.665-16.346], p=0.005). In fact, patients with index surgery complications graded higher than Clavien-Dindo IIIa had increased odds of ileostomy closure problems. This could be explained by the fact that, when abdominal re-exploration is needed after rectal surgery, it is often performed by laparotomy, increasing adhesions that difficult subsequent ileostomy closure. Intriguingly, morbidity of ileostomy closure was not influenced by pre-operative CRP, total protein or albumin.

There are other controversies regarding ileostomy in RC surgery, one of them is timing of ileostomy closure. Previous authors have identified prolonged inter surgery period as associated with an increase in complications of stoma closure [410],[411],[434]. On the contrary, it has also been showed that early closure resulted in more postoperative complications than late one [435]. Others, including a recent metanalysis, however, reported no significant difference in the post-operative morbidity rate, anastomotic

leak, small bowel obstruction, bleeding or ileus between early and late ileostomy reversal. SSI was the only parameter significantly elevated after early closure in comparison with late one [436]–[438]. In our study, time to closure did not impact on the stoma morbidity, closure or related complications [439]. Finally, there was no impact of the anastomotic technique and type of approach (laparoscopic or open) of the index surgery on closure complications.

This study's main limitation is its retrospective nature. However, it is based exclusively on patients treated for RC with a long follow up, offering a perception of the outcomes following ileostomy creation in a real life setting. It is interesting to note that, in a series with 76% of diabetic patients, *Diabetes Mellitus* predicted an increase in complications of ileostomy management, inferior closure rate and increased morbidity of stoma closure. Additionally, morbidity of the initial rectal surgery had a significant impact on the rates of ileostomy closure and associated morbidity.

The difficulty is that many of the patients at high risk of ileostomy complications are also at high threat of anastomotic leak. When deciding over diverting an anastomosis, the influence of predictive factors must be taken into account. Modifiable risk factors like glycaemia control can be improved prior to constructing a derivative stoma. In the particular cases of very advanced disease, considering the negative impact on ileostomy closure rate, one should consider a non-restorative procedure. Nevertheless, if oncologically feasible, the goal is to preserve sphincter function aiming to reduce the rate of unnecessary ileostomies.

CONCLUSION

This study identified *Diabetes Mellitus* and morbidity of the index rectal surgery as factors predictive of ileostomy morbidity, reversal and related complications. In order to decrease morbidity related to loop ileostomy, preoperative optimization of *Diabetes* since rectal cancer diagnosis should be routinely implemented. Also, we must acknowledge the importance of improving the short-term results of the primary surgery in the ileostomy-related outcomes.

It is essential to adjust treatment decisions to patient's predicted morbidity risk and adopt a more selective approach concerning the use of a defunctioning ileostomy, especially for patients in which the risk of having a stoma may offset potential advantages.

Ongoing research: Having identified predictive factors of complications associated with loop ileostomy management and closure, the reality is that many patients still undergo this procedure. The most prevalent complication associated with stoma closure is post-operative ileus that can be related to the defunctioned bowel atrophy. Research is currently ongoing in a study on distal enteric feeding as a protective factor in ileostomy closure in rectal cancer. In this study we hypothesize that distal enteric nutrition can promote bowel trophism and decrease postoperative morbidity (Chapter 6).

Chapter 5

General Discussion and Concluding Remarks

Rectal cancer is a very complex disease with great impact in patients' quality of life. In the past years, significant progresses have been made in the management of RC due to better knowledge of disease pathophysiology, leading to the definition of new therapeutic options. If we consider that there are biological determinants that influence results, we must concede the undeniable impact of the surgical options on the outcomes of patients.

As our goal is to obtain the best possible results minimizing related morbidity, we must search for the biological and surgical determinants of the optimal treatment. This Thesis has tried to gain insight into some specific aspects of RC. A detailed analysis of the results was presented along each publication (Chapter 2, 3 and 4), therefore a general and integrative discussion is presented here.

1. In the article ***Management of rectal cancer: times are changing***, the challenges and recent revolutions in the management of RC were discussed. Preoperative CRT followed by TME has until recently been the state of the art for clinical cT3N+ cases. However, recent studies started to question this classic approach because of a number of issues.

Pelvic MR proved to be the most accurate exam to define locoregional staging with high sensitivity and specificity in the estimation of T and N stages and in the prediction of CRM status prior to surgery. Also, the importance of MR predicted EMVI as a prognostic factor has been acknowledged. CRM involvement is the best predictive tool for both local and distant recurrence, so NCCN and ESMO guidelines now consider not just the TNM classical system for staging but also CRM and EMVI evaluation. In fact, the most significant criteria for the administration of CRT is a predictably threatened or invaded CRM and the presence of EMVI. So, N+ patients can be considered for surgery without neoadjuvant therapy if they present a CRM free of tumour and absence of EMVI. In fact, there are now concerns that, by submitting all cT3N+ tumours to CRT, we might be overtreating some patients and delaying systemic treatment, thereby increasing the risk of distant progression.

On the other hand, the intent of neoadjuvant therapy has expanded, now also with the perspective of achieving a complete response for an organ preservation approach, without resection or just with local resection. In this setting, the indications for neoadjuvant therapy have broadened and this treatment is also considered in patients with T2N0 distally located tumours with risk of sphincter loss or bad functional outcomes. This seems almost a paradox, but it is evolution. On one hand less CRT is given to patients without negative prognostic factors. On the other, to obtain complete responses and avoid mutilating surgeries, we are expanding the indications to the ones with lower tumour stages and even intensifying neoadjuvant regimens.

The recent notion that stage after neoadjuvant CRT more accurately indicates prognosis than pre-treatment clinical one, placed the emphasis on restaging, which gives the indication on the therapeutic conduct to follow. The initial stage is not as relevant as the post treatment one, that expresses tumour biological behaviour. Knowing today that response to therapy is a time-dependent phenomenon, complete

response can be obtained with intensified neoadjuvant regimens and longer waiting time. In carefully selected patients, a Watch and Wait strategy can now be recommended for complete responders, although demanding a very strict surveillance program and consolidation therapies.

Another interesting evolving concept is the importance of good quality surgery. We currently acknowledge the impact of the plane of surgery and mesorectal integrity on patient's outcomes. Also, there has been a change in therapeutic perspective, from one focused on survival and oncological outcomes to one also endorsing quality of life and functional outcomes. In this setting, new technical options are being developed through different surgical approaches, in order to achieve better results and minimize therapeutic morbidity.

Overall, management of RC is clearly going through a significant paradigm change, even conceptual, and it is of paramount importance that patients are referred to specialized Units where these multiple possible strategies can be extensively discussed in a multidisciplinary setting.

2. Many questions are, however, still unanswered. Acknowledging the impact of neoadjuvant radiotherapy on outcomes, the fact is that we rely exclusively on clinical staging to select patients for this therapy. Classical TNM staging system has reached its limit of usefulness encouraging the assimilation of other clinical, pathological and molecular parameters. Nonetheless, so far there are no identified biomarkers predictive of response to CRT and we are still applying it without a real notion of who will respond and who wont. The biological behaviour of RC post CRT has not been unveil and there is an urgent need for biomarkers of response to avoid therapy-related toxicities and overtreatment.

Recognizing the influence of specific miRNAs in colorectal oncogenesis and therapy resistance, the role of these molecules on response to CRT was investigated. The study ***Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma*** showed a statistically significant association of miR-21 overexpression in pre-CRT in RC tissue with worse response to neoadjuvant therapy in the form of LCCRT.

To validate the results of this retrospective study, we conducted a prospective one, now also involving plasma samples. To our knowledge, the study ***Evaluation of Tissue and Circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer*** was the first report in which circulating miR-21 was investigated as a predictive biomarker of response to neoadjuvant CRT in RC. Although here the efficacy of tissue and plasma miR-21 to differentiate responders from non-responders could not be demonstrated, probably due to sample dimension, we did find an increased odds of non-response and recurrence in patients overexpressing miR-21 in pre-CRT tumour tissue. We also observed a decrease in 3-year DFS survival in patients with higher miR-21 levels in pre-CRT tumour tissue and increased odds of pre-CRT circulating miR-21 overexpression in non-responders.

Overall, the concordant results of both these works suggest miR-21 as a biomarker of pathological response to neoadjuvant CRT in RC, predicting good and bad responders. The clinical implications of this idea emphasize the need for larger studies conducted to establish this miRNA definitive role as a predictive tool. Confirmation as such would translate into clinical application through its inclusion in algorithms of treatment decision, certainly allowing a better selection of candidates for CRT.

The easy on obtaining and preserving tissue and plasma samples for miR analysis in the current clinical practice is another motivation to further engage research on this matter.

Before miRNAs become available in the clinical setting, however, it is still necessary to check the best sensitivity and specificity and whether a single or cluster of miRNAs is to be employed. Considering the fact that single miRNAs alone are not considered ideal biomarkers since these molecules are not usually specific for one type of cancer, the combination of different miRNAs and patients clinical parameters such as disease or tumour characteristics could open new possibilities for the prediction of treatment response.

3. During the last 30 years, great surgical progresses were introduced in RC treatment with the objective of improving outcomes and diminishing the morbidity associated with treating this condition. However, RC radical surgery is still related with high rates of anastomotic leak, LARS, suboptimal TME specimens and conversion, with the associated worst prognosis. To overcome the difficulties of distal RC surgery, TaTME was developed in 2010, showing positive short-term results but also bringing new unusual morbidity. Recently, publications raised concerns about the oncological safety of this technique.

In the study ***Transanal Total Mesorectal Excision: 3-year Oncological Outcomes,*** the results of the first 50 TaTME performed in our Unit were analysed. We then intended to analyse data with longer-follow up and also in comparison to a matched lapTME arm. As such, in the study ***Transanal versus Laparoscopic Total Mesorectal Excision. Comparative Study of Long-tem Oncological Outcomes*****,** similar long-term results were obtained, with 2% and 4% LR (p=0.999) in the lapTME and TaTME groups, respectively. Likewise, 4-year OS and DFS were equivalent with 82% and 86% OS (p=0.4), and 91% and 78% DFS (p= 0.1) in the lapTME and TaTME arms, respectively.

Even taking into account the learning curve of TaTME in our Group, the results of these studies showed that TaTME can produce short and long-term oncological safe results. Moreover, they were also similar to our matched lapTME group and compatible to what has been published of the standard of care for mid and low RC.

However, this work emphasizes that this surgical option has a demanding learning curve and significant risk for morbidity. The question is no longer “can good results be obtained by gifted surgeons appropriately trained?” It has moved on to “can this technique be performed reliably, safely and with good outcomes by the average surgeon on the common patient? For its safe implementation it is imperative to fully comprehend the change in anatomical perspective it engages, to implement a structured multimodal learning program, follow international guidelines and employ the technique selectively. Also, it is fundamental that surgeons are experienced not just in laparoscopy but also in single-port and low pelvic surgery. Finally, the transparent scrutiny of the TaTME technique relies in reporting one’s results, participating in ongoing multicentre randomized trials and endorsing international Registries for audited data.

TaTME does not intent to replace other established approaches to rectal surgery but to add new alternatives to address difficult cases. The fact is that, whatever technique is used to performed low RC surgery, it requires advanced surgical skills and optimal results can only be achieved with adequate training and continuous evaluation of outcomes to ensure they improve as experience grows.

4. As we understand TME as one of the greatest revolutions of RC treatment, we also admit the negative impact of this technique on the quality of life of patients with distal RC. In this setting, less aggressive therapeutic strategies started to be discussed, namely neoadjuvant CRT combined with local excision (LE). The study ***Oncological Outcomes of Local Excision Compared with Radical Surgery after Neoadjuvant Chemoradiotherapy for Rectal Cancer: A Systematic Review & Meta Analysis*** intended to compare the outcomes of LE versus radical surgery post CRT. This systematic review and meta-analysis of the current literature suggested the absence of differences in LR, OS and DFS between the two therapeutic options.

These results are explained by the fact that the most relevant determinant of LR and survival is not the baseline staging but the post CRT one, that reflects tumour biologic behaviour. This is the same as saying that following neoadjuvant therapy, therapeutics options ultimately depend on the effectiveness of CRT, evaluated through restaging MR. The surgical determinant is, in fact, the tumour biological response. However, some studies included in the metanalysis were based on initial staging and did not show the post CRT stage.

Overall, this meta-analysis opens debate regarding the requirement for radical rectal excision for low RC especially in those with a good response to CRT. In sum, after CRT, patients with an incomplete response contained in the mucosa or submucosa with negative nodes (ycT1N0) can be an indication for LE. This strategy can also be considered in trial setting or as an option for patients refusing abdominoperineal resection or with significant comorbidity. In our current clinical life, this is particularly important in the elderly for whom radical resection harbours significant morbidity and mortality risk. In case of unfavourable pathology in LE specimen, an immediate completion TME is required. In the presence of a cCR, because post CRT LE has wound healing problems and surveillance is facilitated by the absence of scar, observation alone should be carried out. All other cases imply radical resection.

5. Defunctioning ileostomy performed in TME surgery for rectal tumours has a high morbidity rate, related to ileostomy management itself and to the stoma closure. Although ileostomy does reduce the consequences of a leak, the majority of patients does not face this problem and may be unnecessarily exposed to stoma potential complications. In fact, the ileostomy protective effect needs to be balanced against its morbidity. The problem is that many of the patients at high risk of ileostomy complications are also at jeopardy of anastomotic leak and, until now, we could not predict who would develop stoma-related morbidity. The study ***Loop Ileostomy in Rectal Cancer Surgery: Factors Predicting Reversal and Stoma related Morbidity*** intended to identify risk factors predictive of morbidity, of stoma closure and transformation into a permanent one.

This study identified factors predictive of stoma-related morbidity. In fact, *Diabetes Mellitus* and complications of the index rectal surgery predicted higher ileostomy morbidity, lower reversal and more

closure-related problems, with statistically significant associations. Interestingly, although delaying timing of reversal, adjuvant ChT did not impact on the rate of stoma closure. Also, time to ileostomy closure did not impact on the stoma morbidity, closure or related complications nor there was an influence of the index surgery approach (laparoscopic or open) in closure complications. Intriguingly, morbidity of ileostomy reversal was not influenced by pre-operative CRP, neither total protein nor albumin.

When deciding over diverting a colorectal or coloanal anastomosis, the influence of factors predictive of morbidity must be taken into account. It is essential to individualize treatment decisions and adopt a more selective approach concerning the use of a defunctioning ileostomy, especially for patients in which the risks of having a stoma may offset potential advantages. The relevance of controlling modifiable clinical factors prior to stoma construction but also the importance of the primary surgery outcomes must be emphasized. Optimizing diabetes treatment should be a priority, in order to decrease morbidity related to surgical treatment, and an upfront non-restorative surgery should be considered in patients with advanced malignancy. Finally, some complications of ileostomy management can be prophylactically addressed, avoiding high stoma output with adapted antidiarrheal diet, with electrolyte mix, loperamide and codeine.

In summary, there are many factors influencing the outcomes and that dictate the proper therapeutic conduct to follow in the approach of RC patients. There are biological and surgical determinants of the treatment of this disease that need to be analysed, in order to achieve of the best results with the lowest morbidity.

The role of the microRNA in oncogenic molecular pathways is undeniable as is the influence of miR-21 in the response to CRT. Likewise, the selective choice of particular surgical interventions such as TaTME, LE and defunctioning ileostomy, in different clinical settings, is also critical to obtain the appropriate outcomes.

Many more questions arise and further research must be performed to unravel these complex relationships in such a demanding disease.

Chapter 6

Future Directions and Ongoing Research

The work presented here has developed into other research studies currently being conducted in Hospital Beatriz Ângelo and iMedLisboa, Faculty of Pharmacy. Developed in close relation to the issues approached in this Thesis they are as follows:

1. Metabolism-related hormone modulation of colorectal cancer carcinogenesis, response to treatment and clinical outcomes

In the last decades and in parallel with cancer, obesity has emerged as a global epidemic. Growing evidence supports the role of obesity in CRC development, progression, response to therapy and outcome [440], [441]. Obesity may trigger CRC development through several mechanisms, including modulation of multiple oncogenic molecular pathways, promotion of chronic low-grade inflammation or metabolic de-regulation [442]. Moreover, *in vitro* and *in vivo* studies have shown that obesity can modulate stem cell responsiveness in carcinogenesis [443]–[445]. Therefore, imbalances on metabolism-related hormones such as adipokines (adiponectin, leptin, ghrelin or resistin), insulin, or IGF-1 among others, play a role in CRC development [446]–[448]. For instance, two of the most well studied adipokines in the context of CRC are adiponectin and leptin, which are produced by the adipose tissue and are altered in obesity, but nevertheless have antagonist roles in colorectal tumorigenesis.

Several clinical studies suggest that decreased levels of circulating adiponectin may be an increased risk factor for CRC development [448]–[450]. In fact, adiponectin has been described to modulate several signalling pathways implicated in CRC development mainly via AMPK, such as PI3K/AKT, mTOR or JAK/STAT, and a protective role of adiponectin has been suggested [451]–[455].

In turn, leptin levels appear to have a more oncogenic role being gradually increased during the normal mucosa - adenoma - adenocarcinoma progression, but their correlation with CRC risk is not yet completely clear [456]–[458]. Like adiponectin, leptin also modulates oncogenic signalling pathways and seems to promote cancer cell growth, mobility and invasion [456].

On the other hand, the role of adipokines on cancer stem cells (CSCs) biology and their influence on therapeutics is also a focus, as it has been shown that 5-fluorouracil therapy and CSC survival can be offset by higher leptin levels [451][459], [460]. Tumours are classically a heterogeneous mass, and CSCs, or tumour-initiating cells, are a subpopulation of cells within the tumour niche, that by holding stemness properties sustain cancer progression, re-proliferation, metastization and recurrence [461]–[464]. CRC-CSCs are a dynamic population and are continuously altered by both intrinsic and extrinsic factors [378], [465], displaying high self-renewal capacities, plasticity potential, high resistance to tumour microenvironment stress factors and quiescence. These properties are believed to be responsible for CSC resistance to chemotherapy, cancer relapse and metastization [451]. Noteworthy, both CSC and normal stem cells share common signalling pathways that regulate the fine balance between self-renewal and

differentiation, such as Wnt/β-catenin, Notch or Sonic Hedgehog, and their deregulation is associated with tumour development and progression. Transcription factors from these pathways play important roles in cellular plasticity and in controlling the epithelial-mesenchymal transition, a key step in cancer invasion and metastasis[466].

Several reports have shown that, particularly leptin, beside oncogene activation, also activates NF-kB, Wnt and Notch signalling pathways, which are significantly linked to CRC-CSCs and are critical for the maintenance of stemness traits [467]–[469].

It has been shown CRC-CSCs robustly and selectively express leptin receptor which correlates with cell proliferation [466]. Moreover, these cells present activation of the pluripotency-associated oncogene STAT3 and induction of stemness factors Oct4 and Sox2 [466].

Despite all the promising data, the association between metabolism-related hormones and response to therapy has seldom been studied. Furthermore, the link between these hormones and CSC biology opens new venues to therapeutic strategies, by functioning whether as therapeutic targets or therapeutic agents.

The aim of this study is to explore the role of metabolism-related hormones in RC development and outcome, evaluating their influence on response to neoadjuvant CRT and correlation with CSC markers. This work intents to response to the following: 1) Do leptin and adiponectin levels correlate with response to CRT (TRG) or with CSC markers? 2) Do CSC markers levels correlate with response to CRT (TRG)?

2. Establishment of patient-derived colorectal cancer organoids

It is critical to disclosure the mechanisms leading to tumorigenesis in CRC that may also interfere with patient's response to treatment. Cancer cell line and *in vivo* animal models have been instrumental to dissect the carcinogenesis mechanisms by which CRC is developed, as well as to identify novel therapy targets and potential biomarkers. However, although cell lines harbour unlimited proliferative capacity, they no longer recapitulate the genetic heterogeneity of the primary tumour due to functional and genetic changes induced by artificial culture conditions and by the fact that these cells have been established a long time ago. Animal cancer models also provide important insights into cancer biology but they do not often faithfully mimic the pathogenic processes in patients and their generation is time consuming and expensive. Thus, the development of *in vitro* three-dimensional (3D) culture technologies models has increased recently since these models seem to full impersonate the tumour environment [470].

Organoids are multicellular structures that self-organize into complex organ-like structures and exhibit some of the structural and functional features of the tissue or native organ. These structures can be initiated from single cells or from tissue-derived adult stem cells, cultured long-term and be applied in all cell biology and molecular studies already developed for 2D cell lines [471]. These 3D models can be

cryopreserved, stored in living organoid biobanks and genetically modified, remaining genetically and phenotypically stable [470]. Since organoids represent all cellular components of a native organ, they can be used to model human organ development and various human pathologies, such as cancer. In fact, stem-cell (SC) based organoid technology has been introduced recently to establish long-term cultures of both normal and tumour tissues from colon, liver and breast [472]–[475]. Moreover, organoid cultures can be genetically characterized and used for drug testing of novel anticancer drugs, drug-related toxicity and sensitivity studies, correlating data with the characteristics of the original tumour. In addition, cancer organoids can be used in *in vivo* studies and give rise to histologically matching tumour when injected into mice models [474]. The implementation of these organoids provides a unique model to improve the knowledge of disease mechanisms, progression regenerative and precision Medicine.

RC organoids are an ideal tool to identify and assess the efficacy of new anticancer drug and the most promising technique for future personalized Medicine. Patient-derived organoids (PDOs) have the same phenotype and molecular features of the original tissues and the genotypic profiling of patient-derived CRC organoids are similar to those of the primary tumour [476], [477]. Since these organoids share histological and genetic characteristics with the corresponding patient, they can be used in studies of drug screening and personalized therapy to predict patient response. Each PDO can serve as a small patient trial. Besides, the development of PDOs from normal healthy tissue of the same patient gives the opportunity to develop less toxic drugs by screening for compounds that selectively kill tumour cells without harming the healthy ones. PDOs cultures can be established from tumour biopsies of CRC patients with a success rate of around 70% [476] and are already being applied in drug screening methods. These organoids recapitulate patient clinical responses by predicting whether they will respond to some drugs and if they would not [477].

Throughout this study we intend to contribute for basic and translational research by implementing a 3D model of patient-derived RC organoids. The establishment of a PDOs library will contribute to study tumorigenesis mechanisms, to predict patient's response to treatment and future personalized therapy.

3. Distal feeding as a protective factor in ileostomy closure in rectal cancer

It has been demonstrated that derivative ileostomy decreases the clinical consequences of an anastomotic leak in RC surgery [478]. However, stoma reversal has an overall complication rate up to 20%, with postoperative ileus and surgical site infection, amongst other complications, increasing the length of hospital stay [478]. In RC surgery, the majority of patients has a derivative loop ileostomy for not less than 3 months that is often prolonged in order to complete adjuvant ChT [479]. Defunctioned bowel suffers from atrophy of mucosal and muscular layers becoming unprepared to receive enteric content after stoma closure.

In a preliminary study, it has been demonstrated a decrease in post-operative ileus through daily intestinal administration, through the ileostomy efferent-limb, of 500mL of sodium cloride in the 10 days

previous to stoma closure. These outcomes are currently being validated in a prospective multicentric RCT (NCT02559635) [480].

We hypothesized that distal intestinal stimulation with nutritional formulas previous to stoma closure decreases post-operative ileus and length of hospital stay. Our study is non-randomized with a retrospective historical arm, aiming at determining the potential benefit of intestinal stimulation prior to stoma closure in patients previously submitted to protectomy with derivative ileostomy for non-metastatic RC. In the experimental arm, distal feeding is performed in the 15 days previous to stoma reversal through instillation of 200mL of nutritional supplement in the ileostomy efferent limb.

The primary objective is to analyse the prevalence of post-operative ileus, the secondary aim being the analysis of all other morbidity, including abdominal pain, electrolyte disturbances, food intolerance, nosocomial infections, anastomotic leak and length of hospital stay.

4. Watch and Wait in rectal adenocarcinoma

Current guidelines indicate CRT followed by radical surgery as the standard of care in LARC [17]. Alter neoadjuvant therapy patients are re-staged to reassess response to therapy. This response is variable with 15-30% of patients achieving clinical disappearance of the tumour or cCR, number that is even higher with intensification of neoadjuvant treatment [481]. cCR is associated with improved survival rates in comparison with patients with residual tumour [167]. Some studies point out a progressively better correlation between clinical cCR and pCR [482].

Radical rectal resection with TME is a procedure that, in the majority of patients, is associated with some form of LARS with incontinence, sexual or urinary dysfunction [8],[111]. So, considering the high morbidity associated with TME, the use of this technique to confirm pCR in cCR is increasingly being questioned.

Recent data suggest that patients with cCR can be safely monitored without radical resection, through a rigorous surveillance protocol with periodic digital rectal examination, rectosigmoidoscopy and pelvic MR. This “Watch and Wait” strategy, introduced 30 years ago by a Brazilian group [151],[154] has been sustained by results of other international investigators that present oncological and functional results similar to those obtained by patients treated with the standard surgical strategy [150],[158]. Studies point to the safety of this conservative approach namely in regard to local regrowth and distant spread [163], [386] .

Our study aims to evaluate the oncological and functional outcomes of the active strategy of WW in patients with rectal adenocarcinoma and cCR after neoadjuvant CRT.

The primary objective is the evaluation of OS, DFS, local regrowth, distant recurrence, therapy-related morbidity and quality of life in patients offered WW strategy. The secondary objective is the comparison of the oncological and functional outcomes in patients with cCR treated with “Watch and Wait” to those submitted to TME with pCR. Also, we intent to analyse the correlation between clinical staging through pelvic MR and definitive pathological staging.

5. Intensification of neoadjuvant therapy in patients with rectal adenocarcinoma

Total neoadjuvant therapy (TNT) was developed to obtain higher complete response, ressecability and optimize delivery of systemic therapy to treat micrometastasis [164],[165]. This is done through intensification of neoadjuvant therapy. The addition of ChT before surgery, either before or after CRT, increases the probability of cCR and pCR [161]-[163].

In 2020, the RAPIDO trial [143] compared a conventional arm of CRT followed by TME with an experimental one with SCRT followed by an 18-week period of consolidation ChT and surgery. Disease recurrence, local and distant, and pCR were in favour of the experimental arm. The PRODIGE-23 trial compared conventional CRT followed by TME and adjuvant ChT with intensification of neoadjuvant therapy through FOLFIRINOX followed by CRT and surgery and adjuvant ChT. There was also a higher pCR and DFS rate in the experimental arm. Neither of these trials reports advantages related to OS. In 2020, OPRA trial, that randomized RC patients to induction or consolidation ChT and patients were restaged at 8-12 weeks post CRT, reported higher cCR in the consolidation arm.

Overall, studies seem to show that a TNT and consolidation ChT increases the probability of complete response also improving survival outcomes.

In our study, we hypothesized that cCR rate in patients with LARC is greater with intensification of neoadjuvant therapy in comparison with conventional CRT. This is a prospective unicentric study that intents to evaluate cCR after intensification therapy. This will be in the form of an experimental arm with CRT (with 225mg 5FU /m^2/day or 825mg/m^2/day capecitabine plus 50.4Gy external RT during 5 weeks), followed by 12 weeks of consolidation with 6 cycles of FOLFOX or 4 cycles of XELOX.

The primary objective is to evaluate cCR at 10, 14 and 18 weeks and compare it to the published literature results and to an historical cohort of patients treated with conventional CRT. Secondary goals are to quantify patients that endorse WW strategy as well as analyse OS, DFS, therapeutic toxicity and quality of life.

Chapter 7

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Chapter 8

Addendum

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REVIEW ARTICLE

Management of rectal cancer: Times they are changing

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Abstract Approximately one third of all colorectal malignancies are located in the rectum. It has long been recognized that rectal cancers behave differently from colonic tumors, namely in terms of local recurrence. For this reason, specific protocols have been developed to manage this disease both in staging procedures as well as in neoadjuvant and adjuvant chemoradiation treatments. Magnetic resonance imaging is now obligatory for rectal cancer staging. Also, pre-operative chemoradiation is recommended in the large majority of locally advanced rectal cancers with obvious advantages in downstaging and downsizing tumors, sometimes allowing spincteric-sparing procedures. Total mesorectum excision is now the rule when operating on rectal cancer. Despite these advances, there are still unanswered questions, namely the utility of using neoadjuvant protocols in low lying, early stage tumors with the aim of performing a local excision procedure and the utility of re-staging the disease after neo-adjuvant treatment. In fact, response to neoadjuvant therapy may become a cornerstone of rectal cancer treatment and individualized therapy. Finally, there is the concern that with current protocols, we are overtreating some patients that would not need such extensive treatment.

In this review, we critically examine recent advances in staging, surgery, and chemoradiation in the management of patients with rectal cancer which have not typically been incorporated in published treatment guidelines.

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PALAVRAS-CHAVE
Cancro do reto;
Tratamento multimodal;
Terapêutica individualizada

Manejo do cancro do reto: os tempos estão a mudar

Resumo Cerca de um terço de todos os tumores coloretais estão localizados no reto. Desde há longa data que é reconhecido que os tumores do reto têm um comportamento diferente dos tumores do cólon, nomeadamente em termos de recidiva local. Por este motivo, foram desenvolvidos protocolos específicos para manejar esta doença, tanto em termos de estadiamento como em termos de tratamentos neoadjuvantes e adjuvantes. A ressonância magnética é agora obrigatória como método de estadiamento. Por outro lado, a quimioradioterapia preoperatória é recomendada na grande maioria das neoplasias localmente avançadas com vantagens óbvias no *downstaging* e *downsizing* dos tumores tratados, permitindo por vezes procedimentos cirúrgicos com conservação do aparelho esfincteriano. A excisão do mesoreto é a regra na cirurgia destes tumores. Apesar destes avanços, continuam a existir questões para as quais não existe uma resposta clara, nomeadamente a utilização de protocolos neoadjuvantes em tumores do terço inferior e precoces com o intuito de realizar uma resseção local bem como a utilidade de re-estadiar estes tumores depois da terapêutica neo-adjuvante. De facto, a resposta à terapêutica preoperatória poder-se-á tornar um fator decisivo na implementação de protocolos de terapêutica individualizada. Finalmente, estudos recentes também levantam a questão de alguns dos doentes selecionados para terapêutica neo-adjuvante estarem a ser sobretratados.

Na atual revisão, tentámos rever de forma crítica os avanços recentes utilizados no estadiamento e tratamento destas neoplasias e que atualmente ainda não estão incorporados nas recomendações publicadas.

Introduction

Rectal cancers (RC) comprise approximately 25% of all primary colorectal cancers and follow a different natural disease course compared to colonic tumors. It is well established that surgical approach, local recurrence rates and associated complications of early stage rectal tumors are distinct from colonic cancers. This led to the establishment of specific and distinct protocols for staging and treatment of RC, namely the use of magnetic resonance imaging (MRI) for staging as well as the use of preoperative chemoradiation in selected cases.[1] These advances in the management of patients with RC in the last decade contributed to a marked improvement in patients' outcomes. In the United States five-year survival increased from of 49.2% in the 70s' to 68.5% in the 2000–2005 period. The same trend was observed in Europe.[1–3] This improvement may be related not only to disease detection at an earlier stage and widespread use of optimal surgery with total mesorectal excision (TME) but also to a multidisciplinary approach in specialized centers with an increased use of both radiotherapy and chemotherapy, ideally in a neoadjuvant context.[3,4]

Despite these advances, many issues remain unanswered, namely whether the surgical approach after chemoradiation can be modified based on tumor response, the wait and watch strategy for complete responders and more recently, whether preoperative radiotherapy should be selective, probably based on MRI findings.

In this review, we will review recent changes in the multimodal approach to this tumor.

Tumor staging

Pre operative

Preoperative staging of RC has two main objectives: to define the pertinent anatomy for surgical planning and to determine prognosis. Staging process begins with digital rectal examination. The accuracy of T assessment by digital examination ranges from 58% to 88%, largely depending on the surgeon's experience.[5] For the precise localization of tumors, especially those beyond the reach of an examining finger, rigid proctoscopy is obligatory and should be considered as the single most useful tool.

In the initial preoperative setting, superficial, RCs are probably best staged by endoscopic ultrasonography (EUS), whereas MRI should be used in all other RCs because of its proven high sensitivity and specificity in determining N-stage, extramural vascular invasion (EMVI) and circumferential resection margin (CRM).[6–8] EUS more accurately determines T category as compared to MRI, although low-lying, very high or near-obstrutive tumors are major drawbacks to the use of EUS. Both MRI and EUS share the risk of understaging small lymph nodes (LN) especially when criteria to distinguish inflammatory from pathologic LN rely mainly on size, as many as 25% of positive LNs are smaller than 3 mm.[9] Although not included in TNM classification, tumor proximity to the mesorectal fascia (MRF) increases the risk of compromised CRM (CRM+), which is better predicted by MRI and which has been shown to be an independent risk factor of LR when determined by pathological examination.[10] The MRF with tumor in close proximity

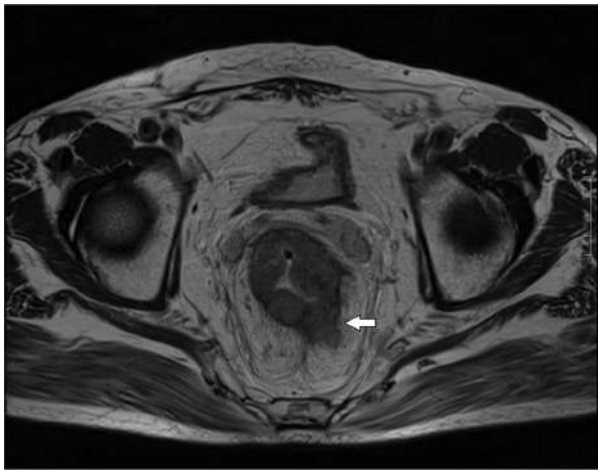

Figure 1 Axial T2-weighted MRI images show rectal tumor. Distance between the tumor and mesorectal fascia (MRF) is less than 1 mm (white arrow) representing a threatened MRF.

(1 mm on MRI) has an increased risk of having a positive CRM as is therefore called a «threatened» MRF (Fig. 1). Recently, the Mercury study was published with the aim of assessing the prognostic relevance of high resolution MRI of CRM.[11] The authors concluded that this staging was superior to AJCC TNM-based criteria for assessing both local and distant recurrence. Accordingly, treatment protocols including preoperative radiotherapy should probably consider these findings. For systemic staging, CT scan of the chest, abdomen, and pelvis is usually sufficient. Thus, in practical terms, it is probably more cost-effective to perform CT scan of the chest (which does not need contrast) combined with abdominal and pelvic MRI. Rectal EUS should only be ordered if pelvic MRI is inconclusive in distinguishing T2 vs. T3N0 tumors. PET-CT imaging cannot be recommended routinely since it only changes patient management in 15% of patients.[12]

Post-treatment staging

As discussed below, tumors staged as T3 N+ or higher are currently managed with neo-adjuvant (CRT). A new concept states that re-staging after CRT might help to identify complete responders and thereby modify treatment and/or surgical strategy.[13] Although this might be debatable, post chemoradiation restaging is a challenge to all imaging modalities due to radiation-induced changes, namely fibrosis, edema, inflammation, and necrosis. The optimal interval between CRT and surgery has not been clearly defined. The Lyon R90-01 study compares a period of less than two weeks with six to eight weeks and found improved T and N downshift with longer intervals.[14] In a recent review from Cleveland Clinic, there was a steep increase in pathologic complete response (pCR) after 7 weeks which reached a plateau only after twelve weeks.[15] Therefore, an interval of seven weeks after CRT but less than twelve weeks is now recommended for post CRT restaging. In respect to the most appropriate imaging, high definition MRI has been shown to accurately distinguish patients with post-treatment tumors confined to the muscularis propria or more superficially (T0-T2N0), from those with more advanced tumors (Fig. 2).[16] Emerging data suggest that reassessment using a combination of high-resolution MRI and diffusion-weighted imaging (DWI), may provide valuable prognostic information before definitive surgery,[17–21] as the latter may distinguish viable tumor from fibrosis or inflammatory from neoplastic LN (Fig. 3).[22]

Surgery

The main aim of surgical treatment of RC is to reduce the risk of residual disease and local relapse while preserving sphincteric, urinary and sexual functions. There are a variety of surgical options in the treatment of RC, which depend not only on tumor location and stage but also on patient sphincter function. Sphincter preservation should not be attempted in those patients with incontinence unless the sphincter can be repaired.

These methods include local procedures, such as transanal local excision and transanal endoscopic microsurgery (TEM) and more invasive procedures involving a transabdominal resection (anterior resection – AR) with colorectal anastomosis, proctectomy with total mesorectal excision (TME) and colo-anal anastomosis or an abdominoperineal resection (APR) with a definitive colostomy.

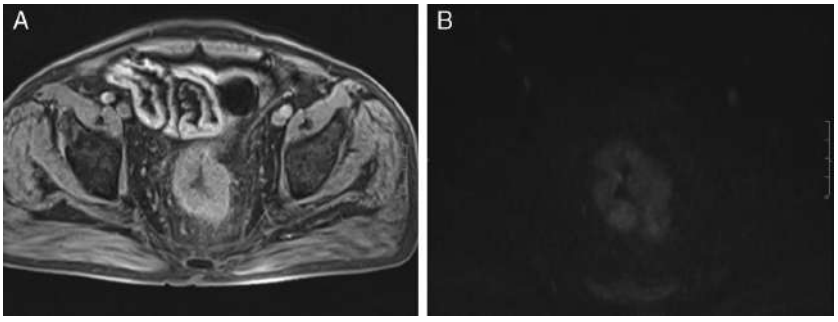

Figure 2 (A) High definition axial T1-weighted MRI post-Gd clearly depicts rectal tumor, with transmural stranding in mesorectal fat. (B) Axial diffusion-weighted at the same level shows hyperintensity of the rectal wall involved by tumor.

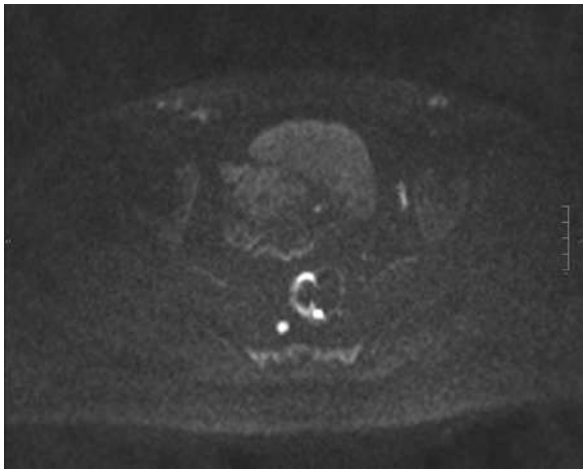

Figure 3 After chemoradiation axial diffusion-weighted shows hyperintensity of the node and rectal wall involved by tumor.

Local excision methods

Local excision methods are performed transanally with a deep margin outside the muscularis propria into the mesorectal fat and a mucosal margin with 1 cm or more around the target lesion. These procedures are reserved for selected cases with a low likelihood of nodal metastasis. This probability depends on the depth of tumor invasion (T stage), tumor differentiation and LVI. For tumors confined to the submucosa, associated nodal metastasis have been reported in 6–11 percent of patients, while cancers invading the muscularis propria have a 10–20 percent risk of nodal metastases and this risk increases to 33–58 percent in tumors extending into the perirectal fat.[23] The incidence of LN metastases also increases dramatically with grade of tumor differentiation with up to 50% of poorly differentiated tumors exhibiting lymph nodes metastasis.[24]

Early RC (confined to the rectal wall without nodal or distant disease – T1N0M0 – with no lymphovascular or perineural invasion, well differentiated and mobile) can be treated with local excision through the ''Parks transanal local excision'' or transanal endoscopic microsurgery (TEM).[25] Parks transanal local excision is appropriate for selected T1N0M0 early RC less than 3 cm in diameter, located in the 8 cm distal rectum, and occupying less than 40% of the circumference of the rectal wall. TEM is a minimally invasive surgical technique originally described by Buess et al. in the 80s',[26–28] which uses a transanal approach with a set of endoscopic surgical instruments that can reach further into the rectum (until 20 cm from the anal verge), along with a form of enhanced or assisted vision.

Both techniques require a full thickness excision performed perpendicularly through the bowel wall into the perirectal fat, with negative (>3 mm) deep and mucosal margins, while avoiding fragmentation (Fig. 4).[25] However, anatomic considerations may prevent local excision even if tumor staging is appropriate. In large lesions, full thickness excision and primary closure can lead to loss of rectal volume and strictures, creating poor functional results particularly if combined with pelvic radiation.

Local therapies are appealing because of their technical ease, low complication rate, rapid post operative recovery with minimal mortality and morbidity, and above all because they avoid the need for a permanent stoma in early, distally located RCs.[26] The major drawback to local procedures, include the absence of pathological staging of nodal involvement, mainly because there is evidence that LN micrometastases also exist in early RC and are unlikely to be identified by endorectal ultrasound. If unfavorable features are observed on pathological examination (high grade, positive or indeterminate margins, perineural or lymphovascular invasion) a radical excision is warranted.[25]

Although more controversial, T2 lesions can also be successfully treated with local excision, especially if combined with neo-adjuvant CRT, although long term outcomes are unknown. The on-going study ACOSOG trial Z6041[29] which is a single-arm study evaluating the oncologic outcome of patients with T2N0M0 distal rectal cancers treated with CRT followed by local excision, may shed some light on this issue. Moreover, the observation that a complete mucosal response often corresponds to negative LNs, also supports the strategy of less aggressive surgical treatments in patients submitted to CRT and with a complete clinical and radiological response.[13] Close follow-up after this strategy is mandatory.

Radical resections

Local recurrence is a major drawback of isolated locoregional treatments such as surgery. In the late 1970s, Heald et al[30] developed the technique of total mesorectal excision (TME) demonstrating that, in some cases, nests of tumor cells outside lymph nodes could be found in the mesorectum and would be left behind by a ''conventional'' anterior resection. Using TME alone, Heald et al.[31] achieved local recurrence rates of less than 5% and emphasis became focused on the CRM.[32–35] Over the last two decades, TME has brought a dramatic improvement in the outcome of surgery for rectal cancer. Anterior resection (AR) is indicated for tumors in the two proximal thirds of the rectum but can also be performed in distal rectal tumors with no involvement of the sphincter. In AR there should be a 5 cm oncological margin from the distal end of the tumor for more proximal tumors but 1–2 cm margins are acceptable for very distal tumors, especially after neo-adjuvant CRT, thereby allowing a sphincter-sparing procedure to be performed. When resection with safe margin carries the loss of continence (direct involvement of the sphincter or levators) or when preoperative continence function is already compromised, an APR is indicated with a definitive colostomy. Although it has been the gold standard of distal rectal cancer surgical therapy, it is nowadays performed in less than 5% of all cases.

Retrospective comparative studies of patients treated with AR and APR, revealed that APR has higher values of local recurrence and reduced survival. This difference in outcome may be explained by the fact that tumors below the peritoneal reflection are usually at a higher stage and have a different lymphatic drainage which might not be included

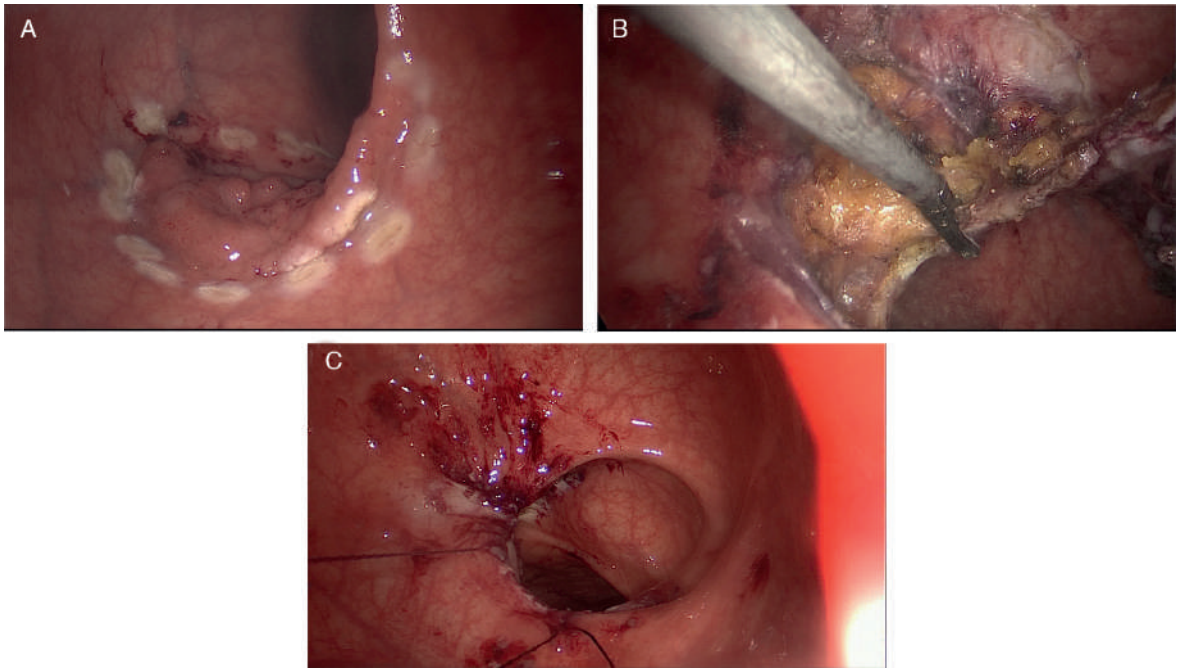

Figure 4 TEM resection of a neoplastic lesion (T1N0) located 20 cm from the anal verge. (A) Delimitation of tumor margins. (B) Full thickness excision performed perpendicularly through the bowel wall into the perirectal fat. (C) Final closure of the operative wound.

in the package of the TME, with higher incidence of lateral pelvic lymph node involvement.[36]

In the distal third of the rectum the mesorectum disappears at the top of the sphincter. Below this level, the sphincter constitutes the CRM. Distal rectal tumors have a shorter distance to cross until the CRM as compared to more proximal tumors, ''protected'' by a thicker mesorectum. Based on the study of the morphometry of the surgical specimen, West et al.[37,38] demonstrated that APR specimens have less tissue volume around the tumor when compared with AR, which was associated with a greater CRM involvement, local recurrence and less overall survival. This problem could be overcome with a ''new'' APR, introduced by Holm et al.,[39] more cylindrical and closer to the original Miles description, with removal of more tissue around the tumor, reducing the probability of CRM involvement.[37,38] This operation involves an abdominal dissection with removal of the rectum and mesorectum down to the levators and a wider perineal dissection, in prone position, with removal of the anal canal, levators and coccyx from below. The perineal defect can be closed with flaps.

Intersphincteric resection

Tumors below 5 cm from the anal verge were not usually considered for a sphincter-sparing surgery because it was not possible to obtain a distal margin of 2 cm through a conventional laparotomy. In this context, the intersphincteric resection (ISR) was introduced as a form of treatment for distal rectal tumors, considering that the mesorectum terminates at the top of the sphincter complex.[40]

ISR is indicated for well differentiated tumors located below 5 cm from the anal verge with predictably negative CRM in MRI. Involvement of the internal sphincter is not a contraindication. In contrast, ISR should not be performed in fixed tumors, involving the external sphincter or levators as well as in patients with poor preoperative continence.[38,40] Limitations for sphincter-sparing procedures are beginning to be regarded as mostly functional and not just oncological.[41,42]

Despite laparoscopic approach of colon cancer is now universally accepted, the extension of this approach to RC is still controversial. There is an evident lack of data and the CLASICC study remains the only randomized controlled multicentre trial comparing the results of classic and laparoscopic approach to rectal cancer surgery. Some groups still express oncological concerns based on the first results of this study, which reported higher rates of CRM involvement and a trend for worst sexual male function in the laparoscopic group. These results were not reproduced and at 3 and 5 years there are no significant differences between both approaches,[43–45] thereby encouraging the use laparoscopic approach in RC.[46]

Chemoradiation treatment

Previous studies have consistently shown that postoperative 5-fluorouracil (5FU)-based chemoradiation significantly improves local control and survival compared with surgery alone.[47–49] When radiotherapy was compared to concurrent CRT, the German Rectal Cancer Trial[50] confirmed that CRT delivered preoperatively, results in a significant decrease

in acute and late toxicities, concomitantly with a better local control of disease and a higher chance of sphincter preservation. Since then, the standard treatment for locally advanced, clinically resectable (T3 and/or N+) rectal cancer is preoperative CRT.

Although 5-fluorouracil continuous infusion (5FU-CI) is the conventional regimen used,[47–49] two recently published studies showed that capecitabine has similar rates of pCR, sphincter-sparing surgery, and toxicity,[51,52] and so both agents can be used in the neo-adjuvant setting.

In patients with pretreatment stage I disease (T2N0), neoadjuvant CRT therapy may be considered in distally located tumors with the aim of downsizing, thereby increasing the chances of a sphincter sparing procedure[53,54]; however the benefits of this strategy remains unproven.

Preoperative vs. postoperative chemoradiation

Two randomized trials compared preoperative vs. postoperative chemoradiation for clinically resectable rectal cancer. The German trial[55] completed the planned accrual of more than 800 patients with rectal cancers less than 16 cm from the anal verge who were randomized to preoperative CRT vs. postoperative CRT. Patients who received preoperative therapy had a significant decrease in local recurrence (6% vs. 15%; $P = .006$), acute toxicity (27% vs. 40%; $P = .001$), and chronic toxicity (14% vs. 24%; $P = .012$) when compared with postoperative therapy. In addition, there was a significant increase in sphincter preservation surgeries (39% vs. 20%; $P = .004$). No differences were observed in 5-year survival. At 10 years the local control benefit of preoperative vs. postoperative therapy was still observed. In contrast to these results which clearly favorable to preoperative treatment, in the NSABP R-03 trial[56] this benefit was not as obvious. However, the results of the NSABP trial should be interpreted with caution because only 267 of the 900 planned patients were accrued, limiting the statistical power to detect differences. Based on these results, preoperative chemoradiation remains the standard of care.

Short-course radiotherapy vs. long-course chemoradiation

The two main strategies of preoperative radiotherapy are long-course chemoradiation and short-course radiation. The first, involves the delivery of a long course of preoperative radiotherapy using conventional doses of 1.8–2 Gy per fraction over 5–6 weeks, with a total dose of 45–50.4 Gy. This approach typically involves the administration of concurrent 5FU or capecitabine-based chemotherapy and is the most accepted approach worldwide.[52]

The rationale for giving chemotherapy concurrently with radiotherapy is that it potentiates local radiotherapy sensitization and has the potential to induce tumor downsizing and/or downstaging, hopefully improving rates of sphincter sparing procedures and increasing rates of pathological complete response (pCR).[36] The second, traditionally used in Scandinavia, consists of short-course preoperative radiotherapy (SCPRT) delivering a total dose of 25 Gy over 5 days (5 fractions) without chemotherapy, followed by surgery within 10 days of the first session of radiotherapy.[57–59] The rationale for this regimen is that the short time period for delivery of the dose may counteract the effects of accelerated cellular repopulation, a phenomenon characteristic of tumor cells exposed to radiotherapy.

In patients with T3/4 rectal cancer, the delivery of a long course of preoperative radiotherapy concurrent with chemotherapy is associated with a relative risk reduction in local recurrence of approximately 50%, whereas short-course radiotherapy does not result in apparent downstaging of tumors in terms of nodal status.[58]

Two large RCT studied the effect of SCPRT in both local recurrence and 5-year survival.[57,58] Although the results of both of these trials favor SCPRT, both were performed before the widespread introduction of TME surgery and, therefore, it remains to be proven whether this beneficial effect would also be observed if TME had been performed.

Therefore on the basis of available evidence, long-course chemoradiation appears preferable, particularly for patients with distal tumors or threatened margins.

Change of surgical strategy based on post-treatment staging

Restaging after neo-adjuvant CRT might help to identify responders to therapy in whom planned treatment based on the original presentation might no longer be indicated. As discussed earlier, the post-treatment assessment often enables sphincter preservation due to tumor downsizing and T or N downshifting.[13] A natural assumption would be that tumors initially staged as T3N0 who after CRT had a downshilt to T0/T1N0 could be safely managed by local excision. Further supporting this practice, in the German Rectal Cancer Study Group,[48–50] the surgeons' pretreatment surgical recommendation was compared with the surgical procedure after neo-adjuvant CRT. Forty percent of patients originally thought to need APR actually underwent a sphincter-preserving procedure without oncologic compromise at a median follow-up of 45 months. However, there are no prospective clinical trials supporting this strategy. The on-going ACOSOG trial Z6041 which is a single-arm study evaluating the outcome of patients with T2N0M0 distal RC treated with CRT followed by local excision procedures, will certainly help to clarify this issue.[29]

CRT causes tumor necrosis, which is then replaced by inflammatory tissue and ultimately fibrosis. Pathologists can quantify the ratio of viable tumor cells to fibrosis to generate a tumor regression grade (TRG).[60] In regard to lymph node response to CRT the only accurate method is pathologic examination of the surgical specimen, but previous observations strongly support the hypothesis that there is a close relationship between primary tumor post-treatment T stage and risk of persistent lymph node metastasis.[61] For this reason, Kosinsky et al.[13] consider that mucosal response can be viewed as a proxy for LN response. Using staging and neoadjuvant CRT protocols discussed above, we may expect rates of pathologic response ranging from 5% to 42%.[62] For this reason, some authors now propose a new algorithm in which surgical approach is based on response to neoadjuvant treatment.[13] Although not validated, it provides a framework for the incorporation of

treatment response in operative planning and sets the stage for considering less radical operative strategies or even a wait and watch strategy in which highly selected RCs are not operated immediately.[13] This strategy is really a «no-immediate» surgical approach, recommended only in highly selected patients who require intensive follow-up with rectal and endoscopic examinations, especially during the first year. Full excisional biopsy is performed in equivocal cases. Disease recurrence in patients previously identified as having had a complete clinical response, requires surgical salvage which has been shown not to compromise outcome as compared with patients who received immediate surgery after neoadjuvant CRT.[63]

Postoperative adjuvant chemotherapy after neoadjuvant chemoradiation and surgery

The neoadjuvant CRT approach commits patients to the entire three component package of CRT, surgery and adjuvant therapy. Beets et al.[53] performed a pooled analysis of 2724 patients who received preoperative chemoradiation. Overall, 41% received postoperative chemotherapy and there was no benefit in disease-free survival in the subset of patients with ypT0N0 or ypT3-4Nx disease. Patients with ypT1-2N0 disease had the greatest benefit, probably because patients who were responders to CRT were also selected. Thus, although its benefit remains controversial, most investigators feel that it is reasonable to use the same adjuvant chemotherapy for colon cancer.[54] For patients selected to receive postoperative adjuvant chemotherapy, 4 months (8 cycles) of FOLFOX/CAPOX or capecitabine monotherapy is recommended although carrying the risk of potentially overtreating some patients.

Novel approaches to neo-adjuvant treatment – the PROSPECT study

As stated before, contemporary management of locally advanced rectal cancer involves preoperative chemoradiation, followed by surgery and then adjuvant systemic chemotherapy. However, although before the advent of TME, LR was a major problem, nowadays the vast majority of rectal cancer deaths are from disseminated metastatic disease, which reinforces the importance of systemic treatment.[64] The problem with the current strategy is that neoadjuvant CRT utilizes either 5-fluorouracil or capecitabine solely as sensitizing agents. Effective chemotherapy with FOLFOX or CAPOX will only start 20–24 weeks from diagnosis, allowing for possible dissemination of micrometastases. As a result, the Alliance for Clinical Trials in Oncology launched the PROSPECT trial early in 2012 – Preoperative Radiation or Selective Preoperative radiation and Evaluation before Chemotherapy and TME,[65] with the aim of moving systemic therapy more proximally in the total treatment course. It is a phase II/III randomized trial to evaluate the impact of selective use of radiation in the era of TME and high-quality MRI imaging. Therefore, in the intervention arm, patients would first start with systemic treatment (FOLFOX $\times$ 6 cycles) with restaging of primary tumor after that. If any progression was observed or regression was lower than 20%, the classic CRT protocol would be performed. If not, the patient would proceed immediately to low anterior resection with TME, eventually followed by an additional 6 cycles. Patients with unexpected positive surgical margins would receive postoperative radiation. This novel approach incorporate selective rather than consistent use of radiation in the treatment of mid RC and customizes subsequent treatment based on response to neoadjuvant FOLFOX.

Conclusions

Multimodal treatment of RC with preoperative CRT in clinical T3N1 cases has improved local recurrence rates and, in the some cases, has allowed a sphincter preservation procedure. TME is now part of an optimal radical resection for RC with the emphasis on CRM.

However, recent studies start to question this classic approach because of a number of issues. First, there is clear evidence that pathologic stage after neo-adjuvant CRT more accurately indicates prognosis than initial clinical stage. However, NCCN and ESMO treatment guidelines, besides not recommending restaging after neoadjuvant therapy, still consider cTNM staging as an indicator for such therapy whereas recent studies demonstrate that high definition MRI with accurate staging of CRM, may be a better predictor for both local and distant recurrence. Also, preoperative CRT might also be considered in patients with T2N0 distally located tumors and, in very carefully selected responders, a wait and watch strategy may be recommended. Finally, there are now concerns that by submitting to CRT all patients clinically staged as T3N+, we might be (i) overtreating some patients (ii) delaying systemic treatment to 4–5 months after diagnosis thereby increasing the risk to systemic dissemination.

Therefore, management of RC is clearly going to change in a near future and it is of paramount importance that these patients are referred to specialized centers where these multiple and possible strategies are extensively discussed in a multidisciplinary team. Gastroenterologists should definitely be part of this team!

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data and that all the patients included in the study have received sufficient information and have given their informed consent in writing to participate in that study.

Right to privacy and informed consent. The authors must have obtained the informed consent of the patients and/or subjects mentioned in the article. The author for correspondence must be in possession of this document.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in rectal Adenocarcinoma

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Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma

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Background: Patients with locally advanced rectal adenocarcinoma (LARC) are treated with neoadjuvant chemoradiotherapy (CRT). However, biomarkers for patient selection are lacking, and the association between miRNA expression and treatment response and oncological outcomes is unclear.

Objectives: To investigate miRNAs as predictors of response to neoadjuvant CRT and its association with oncological outcomes.

Methods: This retrospective study analyzed miRNA expression (miR-16, miR-21, miR-135b, miR-145, and miR-335) in pre- and post-chemoradiation rectal adenocarcinoma tissue and non-neoplastic mucosa in 91 patients treated with neoadjuvant CRT (50.4 Gy) and proctectomy. Two groups were defined: a pathological complete responders group (tumor regression grade—TRG 0) and a pathological incomplete responders group (TRG 1, 2, and 3).

Results: miR-21 and miR-135b were upregulated in tumor tissue of incomplete responders comparing with non-neoplastic tissue ($p = 0.008$ and $p < 0.0001$, respectively). Multivariate analysis showed significant association between miR-21 in pre-CRT tumor tissue and response, with a 3.67 odds ratio (OR) of incomplete response in patients with higher miR-21 levels ($p = 0.04$). Although with no significance, patients treated with 5-fluorouracil (5-FU) presented reduced odds of incomplete response compared with those treated with capecitabine (OR = 0.19; 95% confidence interval (CI) 0.03–1.12, $p = 0.05$). Moreover, significant differences were seen in overall survival (OS) in relation to clinical TNM stage ($p = 0.0004$), cT ($p = 0.0001$), presence of distant disease ($p = 0.002$), mesorectal tumor deposits ($p = 0.003$), and tumor regression grade ($p = 0.04$).

Conclusion: miR-21 may predict response to CRT in rectal cancer (RC).

Keywords: rectal cancer, chemoradiotherapy response, tumor regression grade, miR-21, biomarkers

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent neoplasia in the world, and rectal cancer (RC) corresponded to 30% of all colorectal malignancies in 2019 (1). The current treatment for patients with locally advanced rectal adenocarcinoma (LARC) is neoadjuvant chemoradiotherapy (CRT) in order to achieve downstaging, increase R0 resections, allow sphincter-sparing surgery, and decrease local recurrence (LR) (2). After neoadjuvant treatment, patients are restaged and almost 30% develop clinical complete response (cCR) with no residual tumor identified, 46–60% achieve some degree of tumor downstaging, while 30% exhibit resistance to CRT (3). Non-responders are at increased risk of disease progression and unnecessary toxicity caused by CRT.

Recent data suggest that clinical complete responders can safely undergo a conservative approach without surgery (4). By contrast, the European Society for Medical Oncology (ESMO) guidelines recommend upfront surgery in T3a-bN1 tumors if there is no evidence of involvement of the mesorectal fascia (2). Thus, pretreatment prediction of good and bad responders could be important in deciding whether the patient should or not undergo neoadjuvant CRT. Currently, although molecular heterogeneity is a well-recognized feature of most tumors, CRC patients are still treated based solely on clinical stage. The inclusion of molecular markers in a treatment algorithm could potentially stratify patients and thus allow a better choice of candidates. No biomarkers are yet validated for selection of patients for CRT.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs that act as post-transcriptional regulators binding a variety of messenger RNA targets, inhibiting its translation. Although the precise biological role of many miRNAs is yet to be entirely elucidated, up to 30% of the human genome is regulated by these molecules through influence in relevant cellular functions, including stress responses, angiogenesis, metastasis, and programmed cell death (5). Carcinogenic pathways are regulated by miRNAs and their potential role in oncogenesis raised the possibility of being used as biomarkers in cancer treatment response or prediction of prognosis (6).

Although most published data is on colon cancer, some studies have addressed RC differentiating the miRNAome between these two malignancies. Moreover, specific miRNAs have been proposed as predictors of response to CRT in RC although with some inconsistent findings (7–11). These results need to be validated and are mostly related to 5-fluorouracil (5-FU)-based therapies, not much being known about miRNAs as biomarkers of response to capecitabine.

This study aimed to investigate miRNAs as predictors of pathological response to CRT in RC. Based on literature review including our own previously published data (12), five miRNAs were chosen by virtue of having been demonstrated to be potential biomarkers for CRC. Thus, miR-16, miR-21, miR-135b, miR-145, and miR-335 expression was determined and correlated with pathological response and oncological outcomes.

MATERIALS AND METHODS

Patients and Tissue Samples

This was a retrospective study of prospectively analyzed data and samples. Patients with RC (stages I–IV, American Joint Committee on Cancer, AJCC) diagnosed between March 2013 and September 2017 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal) treated with long course CRT and proctectomy were eligible.

Patients had a preoperative staging with pelvic magnetic resonance (MR), thoraco-abdomino-pelvic computed tomography (CT), and endoanal ultrasound when pelvic MR was not clinically possible. Histopathological features were confirmed by pathological analysis and patients were staged according to TNM staging system (8th edition, 2017). Patients with other histological types of rectal malignancy, not submitted to CRT or surgical resection, pregnant, or under the age of 18 were excluded.

Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institution's Human Research Committee and Ethical Committee on March 13, 2017. The study was registered in the Portuguese Data Protection Agency.

Neoadjuvant Treatment

All patients underwent neoadjuvant CRT consisting of a 2-Gy daily fraction of pelvic irradiation, 5 times a week, in a total of 50.4 Gy. Radiation was delivered with capecitabine (825 mg/m^2/day) or 5-FU (1,000 mg/m^2/day on days 1–5 and days 29–33). All patients except for one received more than 80% of the planned radiotherapy with a curative intent. Surgery was performed 10–12 weeks after CRT.

Assessment of Pathological Response

Pathology specimens were graded by tumor regression grade (TRG) according to the College of American Pathologists guidelines (CAP, TNM 7th edition). TRG was assessed by two pathologists, blinded to patients clinical data, and categorized as TRG 0 (no viable tumor cells or complete response), TRG 1 (single cells or little groups of cancer cells), TRG 2 (residual cancer outgrown by fibrosis), and TRG 3 (minimal or no tumor kill with extensive residual cancer). Tissue was retrieved from formalin-fixed paraffin embedded (FFPE) samples. Histological confirmation of the biopsy samples was done by pathologist review, and neoplastic and adjacent non-neoplastic rectal tissues were differentiated based on hematoxylin and eosin (H&E) stain. A fixed amount of tissue (80 μm) across the samples was extracted for RNA isolation. Pre-CRT RC biopsies (colonoscopy) were obtained from complete and incomplete responders as well as post-CRT tumor tissues (protectomy specimen) from incomplete responders. To allow a direct comparison of RC to matched non-neoplastic rectal mucosa, we collected adjacent (>1 cm distant) non-tumor tissue in both biopsies and

TABLE 1 | Patient clinical parameters.

Clinical parameters		Patients ($n = 91$)
Gender, *n* (%)	Male	60 (66)
	Female	31 (34)
Age, median		68 (45–83)
BMI, median		26 (15–45)
ASA score, *n* (%)	Not discriminated	11 (12)
	I	2 (2)
	II	56 (62)
	III	21 (23)
	IV	1 (1)
Grade	G1/G2	85 (93)
	G3/G4	6 (7)
Location (%)	1/3 superior	19 (21)
	1/3 medium	28 (31)
	1/3 inferior	44 (48)
Tumor extension (mm), median		58 (5–120)
Distance to anal verge (mm), median		60 (0–130)
cT	1	1 (1)
	2	10 (11)
	3	64 (70)
	4	16 (18)
cN	0	9 (10)
	+	82 (90)
cM	0	78 (86)
	1	13 (14)
CRM, *n* (%)	Free	67 (74)
	Threatened or invaded	24 (26)
EMVI, *n* (%)	Negative	86 (95)
	Present	5 (5)
c Stage, *n* (%)	I	3 (3)
	II	8 (9)
	III	68 (75)
	IV	12 (13)
CEA (mg/mL)		1.9 (0.5–163)
Chemotherapy	Capecitabine based	83 (91)
	5-FU based	8 (9)
TRG (CAP), *n* (%)	0	15 (17)
	1	24 (26)
	2	33 (36)
	3	19 (21)

BMI, Body Mass Index; ASA, American Society of Anaesthesiologists; CRM, circumferential resection margin; EMVI, extramural vascular invasion; CEA, carcinoembrinonary antigen; TRG, tumor regression grade; CAP, College of American Pathologists.

protectomy specimens. Two groups of patients were defined, including a pathological complete responders group (TRG 0) and a pathological incomplete responders group (TRG 1, 2, and 3).

RNA Isolation

For total RNA isolation, pre- and post-CRT FFPE non-neoplastic and tumor rectal tissue samples were first deparaffinized with xylene (VWR International, Radnor, PA, USA) in two washing steps at 50°C. The samples were then fully homogenized into fine particles in 100% ethanol using a motor-driven grinder and centrifuged at maximum speed for 5 min. The collected pellet was rehydrated with 95% ethanol for 10 min following a new centrifugation step at maximum speed for 5 min. Then, samples were lysed with 500 μg/mL proteinase K in 100 μL of protease digestion buffer (20 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$ 0.5% SDS) at 55°C. Total RNA was isolated using Ribozol™ reagent (VWR International, Radnor, PA, USA) according to the manufacturer's instructions and eluted into 20 μL RNase-free water. For a better evaluation of miRNAs quantity in total RNA, the miRNA concentration was determined using Qubit™ miRNA Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

Expression Analysis by Real-Time PCR (RT-PCR)

cDNA synthesis was performed using TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For a uniform quantification of the quantity of miRNA to be used in cDNA, 2 μL of total RNA (corresponding to 2 ng of RNA) was extended by a 3′ poly-A tailing reaction and a 5′ adaptor ligation to the mature miRNAs. miRNAs were reverse transcribed into cDNA by reverse transcription using Universal RT primers. In order to improve detection of low-expressing miRNA targets, a pre-amplification of the cDNA was performed using the Universal miR-Amp Primers and miR-Amp Master Mix to uniformly increase the amount of cDNA for each target, maintaining the relative differential expression levels. cDNA samples were stored at −20°C. Real-time polymerase chain reaction (PCR) was performed on a Quantstudio™ 7 Flex real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan™ Advanced microRNA Assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) to assess the expression profile of hsa-miR-16-5p (Assay ID 477860_mir), hsa-miR-135b-5p (Assay ID 478582_mir), hsa-miR-145-5p (Assay ID 477916_mir), hsa-miR-335-5p (Assay ID 478324_mir), and hsa-miR-21-5p (Assay ID 477975_mir). All reactions were performed in duplicate.

Due to the fact that a consensual endogenous control for miR expression in rectal tissue has still not been determined, initial preliminary analyses were performed to test several miRNAs as controls. Normalization was then performed with hsa-miR-484 (Assay ID 478308_mir), identified as the most stably expressed miRNA with the lowest expression variability between samples in these patient data set when compared with mir-1228-5p, miR-345-5p, and miR-103a-3p and the small nuclear (snRNA) U6 and RNU6B, some considered controls for CRC tissues. Expression levels were calculated by the threshold cycle ($2^{-\Delta\Delta Ct}$ method) where $\Delta\Delta Ct$ = (Ct target miR − Ct control) sample − (Ct target miR − Ct control) median, when amplification values were detected in the real-time PCR. Due to lack of amplification values detected by the real-time PCR in all patient tissues,

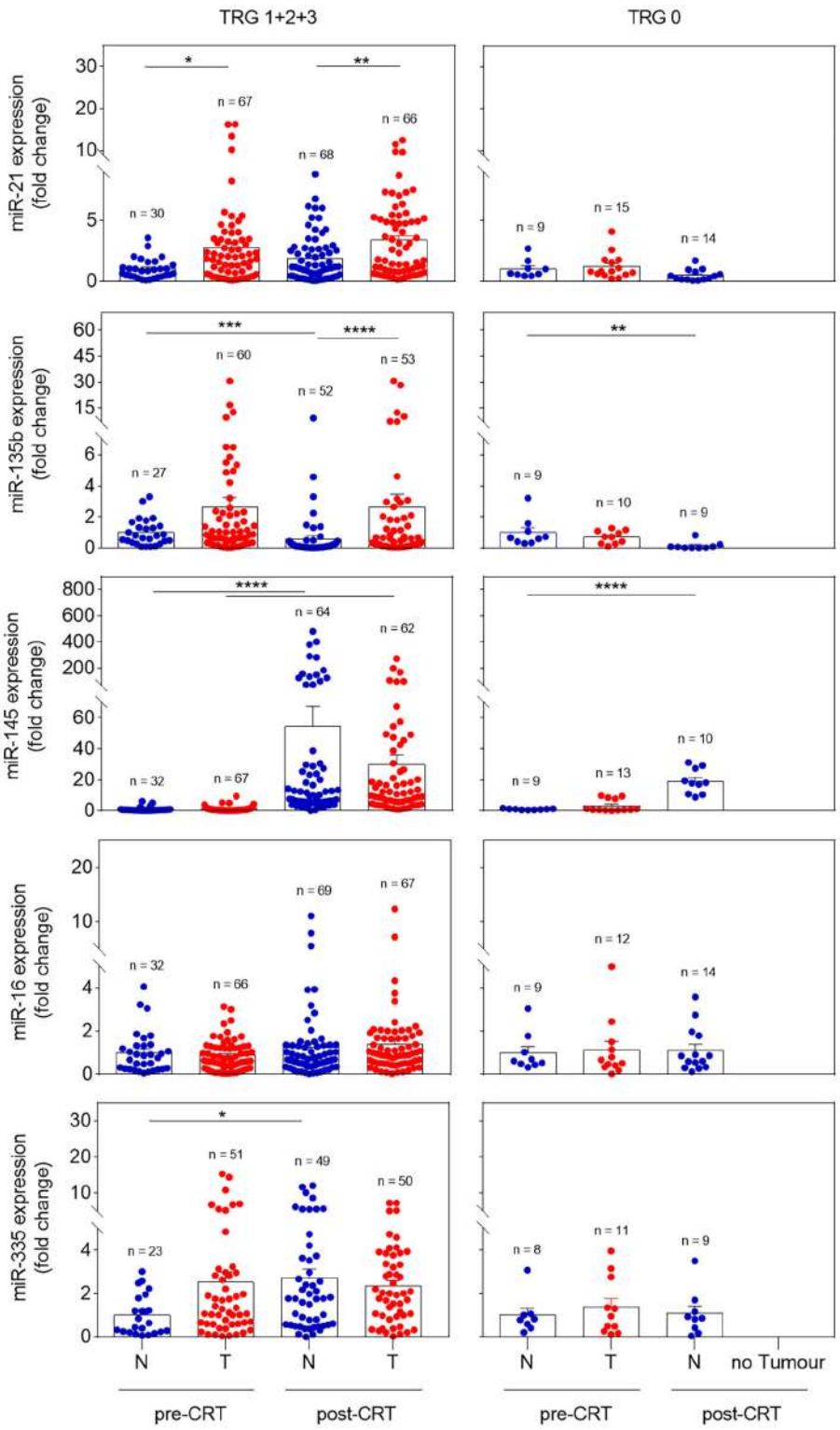

FIGURE 1 | Expression profile of miR-21, miR-135b, miR-145, miR-16, and miR-335 in pre- and post-CRT non-neoplastic and tumor tissues in incomplete (TRG 1 + 2 + 3) and complete responders (TRG 0). Pre-CRT non-neoplastic tissue samples used in this study were derived from a maximum of 37 and 10 patients in TRG 1 + 2 + 3 and TRG 0 groups, respectively. Pre-CRT tumor tissue and post-CRT tissue samples were analyzed from a maximum of 76 patients (TRG 1 + 2 + 3) and 15 patients (TRG 0). Data are mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$), in which N corresponds to non-neoplastic tissue and T to tumor tissue.

TABLE 2 | Association between miRNA expression and TRG.

Variables		OR	95% CI	*p*-Value
miR-21	≤0.66	1.00		
Pre-CRT non-neoplastic	>0.66	1.428	0.32–6.79	0.6407
miR-21	≤1.18	1.00		
Pre-CRT tumor	>1.18	3.58	1.13–12.65	0.0346
miR-135b	≤0.8	1.00		
Pre-CRT non-neoplastic	>0.8	1.85	0.40–10.27	0.4420
miR-135b	≤1.01	1.00		
Pre-CRT tumor	>1.01	2.33	0.58–11.62	0.25
miR-145	≤1.28	1.00		
Pre-CRT non-neoplastic	>1.28	0.65	0.11–5.18	0.643
miR-145	≤0.73	1.00		
Pre-CRT tumor	>0.73	0.88	0.26–3.02	0.838
miR-16	≤0.77	1.00		
Pre-CRT non-neoplastic	>0.77	2.00	0.44–10.80	0.3806
miR-16	≤0.54	1.00		
Pre-CRT tumor	>0.54	1.75	0.49–6.19	0.375
miR-335	≤1.16	1.00		
Pre-CRT non-neoplastic	>1.16	4.5	0.64–91.58	0.191
miR-335	≤1.01	1.00		
Pre-CRT tumor	>1.01	1.86	0.49–7.24	0.354

Simple logistic regression using miRNA dichotomized according to cut-offs determined with ROC curve analysis. OR, odds ratio of incomplete/non-response; CI, confidence interval.

a variable number of samples were included in each miRNA expression profile.

Statistical Analysis

The estimated sample size was 86 patients (43 patients per group of low and high miR expression). Sample size was calculated with an estimated proportion of patients TRG 0 with high and low miR-21 expression of 0.067 and 0.35, respectively. Type I and II errors were set at $\alpha = 0.05$ and $\beta = 0.2$, respectively. miRNA expression was analyzed using the GraphPad Prism software package, version 7.0 (GraphPad software Inc., San Diego, CA, USA). Normal distribution was determined using the D'Agostino and Pearson omnibus test. Data was analyzed according to normality of values distribution using the one-way analysis of variance (ANOVA) followed by Kruskal–Wallis non-parametric Dunn's multiple comparison test or ANOVA Tukey's multiple comparisons test according to Gaussian distribution.

Receiver operating characteristic curve (ROC) analysis was then conducted, establishing the optimal cutoffs for each miRNA before CRT in non-neoplastic and tumor tissue, determined as the point closest to the top left part of the plot with perfect sensibility and sensitivity. All miRNAs were dichotomized according to these cutoffs. Further analysis was also performed to explore the best discriminative cutoff point for miR-21 by comparing the cutoff determined in this study (1.18) with the previously reported miR-21 cutoff (2.8) (13). Both cutoffs presented a similar area under the curve (AUC), with our cutoff having an AUC value of 0.65 (95% CI = 0.518–0.790), a higher specificity (66 vs. 60%), a lower sensitivity (64 vs. 87%), a similar positive predictive value (PPV) (92 vs. 90%) and a lower negative predictive value (NPV) (29 vs. 43%) (**Supplementary Figure 1** and **Supplementary Table 1**). Although both dichotomizations presented similar performance, we chose the cutoff determined in this study that yielded a better-distributed categorization of miR-21.

Simple and multiple logistic regressions were used to correlate each variable with the outcome response after CRT: "pathological complete response (TRG 0)" or "pathological incomplete response (TRG 1, 2, and 3)." For continuous variables, linearity of the logit in the predictor was assessed using a cubic spline and Wald test of linearity.

The association between high and low miR-21 expression and clinical characteristics was tested with chi-square test. Only variables with $p \leq 0.25$ in simple logistic regression or considered clinically relevant were selected to multiple logistic regression. Multicollinearity was also analyzed through the observation of variance inflation factors. A stepwise both-selection technique was used to create the multiple regression model. ROC curve was computed and the respective AUC was calculated to assess discriminatory ability of the model.

RESULTS

Patient Clinical Parameters

Demographic and clinical parameters of the 91 patients are summarized in **Table 1**. With 4 patients lost (4.4%), median follow up was 4.2 years.

miRNA Expression in Complete and Incomplete Responders

miRNA expression profiles were analyzed in non-neoplastic and tumor rectal tissue before and after CRT in all 91 patients. Significant changes were observed when comparing incomplete and complete responders (**Figure 1**). In incomplete responders, miR-21 revealed higher expression in pre-CRT tumor tissue in comparison with non-neoplastic tissue ($p = 0.03$). Post-CRT samples also presented higher levels of miR-21 in tumor tissue ($p = 0.008$). In contrast, in complete responders, miR-21 showed similar levels in pre-CRT tumor and non-neoplastic tissue.

miR-135b presented a profile equivalent to miR-21. In incomplete responders, miR-135b upregulation was detected in tumor tissue, either pre- or post-CRT ($p < 0.0001$), whereas in complete responders equal levels were found in pre-CRT tumor samples and non-neoplastic tissue. Although miR-145 expression showed significant differences among pre- and post-CRT non-neoplastic and tumor tissues ($p < 0.0001$) in incomplete responders, similar results were detected in complete responders, suggesting a lack of discriminative value of this miRNA.

Moreover, there were no significant differences in miR-16 and miR-335 expression between groups. Thus, these results suggest that miR-21 and miR-135b might be useful biomarkers to predict treatment response.

Identification of miRNAs Involved in TRG

The significantly different expression of miRNAs between incomplete (TRG 1, 2, and 3) and complete responders (TRG

TABLE 3 | Clinical parameters and TRG in miR-21 expressing patients.

Simple logistic regression		TRG 0 $n = 15$	TRG 1 + 2 + 3 $n = 67$	OR	95% CI	p-Value
Continuous variables		**Median (Max–Min)**	**Median (Max–Min)**			
Age		67.0 (53–81)	68 (45.0–83)	1.00	0.94–1.06	0.976
Weight		70.0 (45–113)	68 (44.0–119)	0.99	0.96–1.03	0.645
BMI		25.0 (19–41)	26 (15.0–45)	1.00	0.91–1.13	0.921
Tumor extension (mm)		54.5 (21–110)	56 (5–120)	0.99	0.97–1.03	0.901
CEA		2.8 (0.5–8.3)	1.9 (0.5–163)	1.07	0.99–1.29	0.299
Weeks post-chemo		11 (7.0–28)	10 (2.0–21)	0.87	0.73–1.01	0.081
Categorical variables		**Number**	**Number**			
Gender	Male	11	45	1.00		
	Female	4	22	1.34	0.41–5.29	0.643
Tumor location	0	3	14	1.00		
	1	8	16	0.43	0.08–1.81	0.271
	2	4	37	1.98	0.35–10.13	0.407
ASA	1 + 2	9	54			
	3 + 4	6	13	0.36	0.11–1.24	0.0955
CRM MR	Free	11	50		1.00	
	Threatened	1	4	0.88	0.12–18.11	0.913
	Invaded	3	13	0.95	0.25–4.66	0.947
Extramesorectal nodes	Negative	12	43	1.00		
	Positive	3	24	2.23	0.63–10.50	0.247
cT	1 + 2	1	8	1.00		
	3 + 4	14	59	0.53	0.03–3.23	0.561
cN	0	2	6	1.00		
	1	13	61	1.56	0.21–7.721	0.608
cM	0	14	57	1.00		
	1	1	10	2.46	0.42–46.96	0.41
Stage	I	1	2	1.00		
	II	2	5	1.25	0.04–23.53	0.880
	III	11	51	2.32	0.10–26.38	0.508
	IV	1	9	4.50	0.14–156.82	0.352
Stage	I + II	3	7	1.00		
	III + IV	12	60	2.14	0.42–8.99	0.315
Chemotherapy	Capecitabine	12	64	1.00		
	5-FU	3	3	0.188	0.03–1.12	0.05

Simple logistic regression analysis using TRG as dependent variable and clinical/molecular variables as independent variables. From the initial group of 91 patients, 82 expressed miR-21.

TRG, Tumor regression grade; OR, odds ratio of incomplete response; CI, confidence interval; BMI, body mass index; CEA, carcinoembrionary antigen; ASA, American Society of Anaesthesiologists; CRM, circumferential resection margin; MR, magnetic resonance.

0) suggested a possible association between miRNA expression and treatment response. The relation between miRNA in pre-CRT samples and response was analyzed with logistic regression (**Table 2**). A significant association was found between miR-21 in pre-CRT tumor tissue and TRG. Patients with expression higher than 1.18 (fold change) were 3.58 more likely to obtain an incomplete response than those with expression lower than 1.18 ($p = 0.03$). However, there was no association between pre-CRT non-neoplastic or tumor tissue expression of miR-135b and TRG. The same was found for miR-16, miR-145, and miR-335. Given the association of miR-21 and response, we proceeded with the study of this miRNA.

Clinical Parameters and TRG in miR-21 Expressing Patients

From the initial group of 91 patients, only 82 patients expressed miR-21 due to lack of amplification. Although with no significant association between type of radio-sensitizing agent and TRG, patients treated with 5-FU presented reduced odds ratio (OR) of incomplete response compared with patients treated with capecitabine [OR = 0.19; 95% confidence interval (CI) 0.03–1.12, $p = 0.05$]. It was also recognized a definitive trend toward reduced odds of incomplete response with longer waiting times (OR = 0.87; 95% CI 0.73–1.01, $p = 0.08$). However, there was no association between patient gender, age, weigh, American Society of Anaesthesiologists (ASA) score, body mass index

TABLE 4 | Clinical parameters and levels of miR-21 expression.

Variables		Number (%)	High miR-21	Low miR-21	*p*-Value
miR-21 pre-CRT tumor		82 (100)	48 (58.5)	34 (41.5)	
Age	<60	15 (18.3)	7 (14.6)	8 (23.5)	0.302
	≥60	67 (81.7)	41 (85.4)	26 (76.5)	
Sex	Male	56 (68.3)	32 (66.7)	24 (70.6)	0.707
	Female	26 (31.7)	16 (33.3)	10 (29.4)	
BMI	Low weight	1 (1.2)	0 (0)	1 (2.9)	0.236
	Normal	27 (32.9)	17 (35.4)	10 (29.4)	
	Pre-obesity	39 (47.6)	25 (52.1)	14 (41.2)	
	Obesity	15 (18.3)	6 (12.5)	9 (26.5)	
ASA score	1	2 (2.4)	1 (2.1)	1 (2.9)	0.330
	2	53 (64.6)	29 (60.4)	24 (70.6)	
	3	18 (22)	11 (22.9)	7 (20.6)	
	4	1 (1.2)	1 (2.9)	0 (0)	
	ND	8 (9.8)	7 (14.6)	1 (2.9)	
Stage pre-CRT	I	3 (3.7)	1 (2.1)	2 (5.9)	0.720
	II	7 (8.5)	4 (8.3)	3 (8.8)	
	III	62 (75.6)	36 (75.0)	26 (76.5)	
	IV	10 (12.2)	7 (14.6)	3 (8.8)	
Stage post-CRT	0	12 (14.6)	6 (12.5)	6 (17.6)	0.607
	I	6 (7.3)	4 (8.3)	2 (5.9)	
	II	6 (7.3)	5 (10.4)	1 (2.9)	
	III	9 (11.0)	4 (8.3)	5 (14.7)	
	IV	3 (3.7)	1 (2.1)	2 (5.9)	
	NA	5 (6.1)	4 (8.3)	1 (2.9)	
	ND	41 (50)	24 (50.0)	17 (50.0)	
Grade pre-CRT	Low	77 (93.9)	45 (93.8)	32 (94.1)	1.00
	High	5 (6.1)	3 (6.2)	2 (5.9)	
cT	1	1 (1.2)	1 (2.1)	0 (0.0)	0.852
	2	8 (9.8)	5 (10.4)	3 (8.8)	
	3	59 (72.0)	34 (70.8)	25 (73.5)	
	4	14 (17.1)	8 (16.7)	6 (17.6)	
cN	0	8 (9.8)	4 (8.3)	4 (11.8)	0.606
	1	74 (90.2)	44 (91.7)	30 (88.2)	
cM	0	71 (86.6)	41 (85.4)	30 (88.2)	0.712
	1	11 (13.4)	7 (14.6)	4 (11.8)	
pTRG	TRG 0	15 (18.3)	5 (10.4)	10 (29.4)	0.064
	TRG 1	21 (25.6)	16 (33.3)	5 (14.7)	
	TRG 2	32 (39.0)	20 (41.7)	12 (35.3)	
	TRG 3	14 (17.1)	7 (14.6)	7 (20.6)	
Distant recurrence	No	60 (73.2)	33 (68.8)	27 (79.4)	0.283
	Yes	22 (26.8)	15 (31.2)	7 (20.6)	
Local recurrence	No	75 (91.5)	43 (89.6)	32 (94.1)	0.694
	Yes	7 (8.5)	5 (10.4)	2 (5.9)	
Death	No	61 (74.4)	33 (68.8)	28 (82.4)	0.164
	Yes	21 (25.6)	15 (31.2)	6 (17.6)	

From the initial group of 91 patients, 82 expressed miR-21.
ASA, American Society of Anaesthesiologists; BMI, body mass index; CRT, chemoradiotherapy; pTRG, pathological tumor regression grade.

(BMI), tumor location, tumor extension, histological grade, pre-therapeutic carcinoembrionary antigen (CEA), radiological involvement of the circumferential resection margin (CRM), presence of extramural vascular invasion (EMVI), mesorectal deposits (N1c), extramesorectal nodes, cT, cN, cM, stage (TNM, AJCC), and TRG (**Table 3**).

TABLE 5 | Association between clinical parameters and TRG.

Variables		OR	95% CI	*p*-Value
Stage	1 + 2	1.00		
	3 + 4	2.16	0.388–10.16	0.341
miR-21	≤1.18	1.00		
	>1.18	3.67	1.126–13.49	0.036
ASA score	1 + 2	1.00		
	3 + 4	0.33	0.090–1.185	0.082

Multiple logistic regression analysis using TRG as dependent variable and disease stage, miR-21 and ASA score as independent variables.
OR, odds ratio; CI, confidence interval; ASA, American Society of Anaesthesiologists.

Clinical Parameters and Levels of miR-21 Expression

Although no statistically significant association between clinical parameters and expression of miR-21 was observed, a near significant association was established between this miRNA and TRG, with higher proportion of incomplete response in patients with higher miR-21 levels ($p = 0.06$) (**Table 4**). In multivariate analysis, after adjustment for clinically and statistically relevant variables (disease stage and ASA score), this association was again demonstrated with odds of incomplete response 3.67 times greater in individuals with a miR-21 overexpression (>1.18-fold change) when compared with those with lower miR-21 levels (≤1.18-fold change) (95% CI 1.13–13.5; $p = 0.04$) (**Table 5**).

Oncological Outcomes

Overall survival (OS) at 2 and 5 years was 90% (95% CI 83.4–96.9) and 72% (95% CI 61.6–85.1), respectively. Overall disease-free survival (DFS) at 2 and 5 years was 74.1% (95% CI 64.4–84.8) and 66% (95% CI 55–80), respectively (**Figure 2**).

Overall survival was not influenced by age, gender, tumor location, grade, mesorectal nodes, extramesorectal nodes, type of radio-sensitizing agent, post-operative complications, and levels of miR-21 ($p = 0.36$) (**Figure 3** and **Supplementary Figure 2**). As expected, there was an impact in OS in relation to T ($p < 0.0001$) mesorectal tumor deposits, N1c ($p = 0.003$), distant metastasis M ($p = 0.002$), stage ($p = 0.0004$), and TRG ($p = 0.04$) with a borderline significance for threatened circumferential resection margin, CRM ($p = 0.05$) (**Figure 3**). Also, there was increase death risk in individuals with higher cT (HR = 4.78; 95% CI 1.96–11.66, $p = 0.0006$), higher stage (HR = 11.1; 95% CI 1.34–91.88, $p = 0.03$), threatened mesorectal fascia (HR = 4.24; 95% CI 1.19–15.08, $p = 0.03$), positive N1c (HR = 5.47; 95% CI 1.56–19.14, $p = 0.008$), distant metastasis (HR = 3.78; 95% CI 1.52–9.4, $p = 0.004$), and TRG 3 (HR = 3.25; 95% CI 0.83–12.71, $p = 0.08$). No association was, however, established between miR-21 expression and risk of death (**Table 6**).

Finally, the utility of miR-21 as a predictor of survival was investigated. The model of prediction, in multivariate analysis, adjusted to the most relevant clinical variables, did not show a significant association between risk of death and higher miR-21 expression (HR = 2.68; 95% CI 0.86–8.36, $p = 0.09$) (**Table 7**).

DISCUSSION

Rectal cancer (RC) patients treated with CRT urgently need biomarkers to distinguish responders from non-responders and allow individualized treatment, with non-responders avoiding neoadjuvant therapy and complete responders eluding mutilating resections. In this work, we investigated five miRNAs as biomarkers to predict response to CRT in RC.

miR-145 and miR-335 are acknowledged to act as tumor suppressor genes (14, 15) and miR-145 is overexpressed in post-CRT tumor tissue in comparison with pre-CRT with significant correlation with tumor regression (7). In our work, no differences were detected in these miRNAs before and after CRT and no correlation was found with response. In addition, miR-16 has been described as a tumor suppressor with downregulation predicting poor prognosis in CRC (16). In our study, miR-16 was not a predictor of response either. miR-135b is an oncomiR that often mediates CRC genes whose overexpression has been correlated with tumor stage and poor clinical outcome (17). We have further analyzed its potential as predictor of response to CRT and found significant differences in expression. In incomplete responders, higher miR-135b levels were found in both pre- and post-CRT tumor tissues comparing with non-neoplastic tissues, whereas in complete responders similar expression was obtained in all samples. We could not, however, correlate miR-135b expression with clinical parameters or TRG.

Finally, in our study we found that incomplete responders had higher miR-21 expression in tumor tissue in comparison with non-neoplastic tissue in both pre- and post-CRT samples. In contrast, complete responders had similar levels in all samples. Moreover, an association was discovered between pre-CRT tumor miR-21 levels and TRG, with a 3.67 odds of non-response in patients with expression higher than 1.18 ($p = 0.04$). Higher miR-21 expression in the tumor prior to treatment was indicative of a worst response. As expected, OS was influenced by cT, cM, N1c, TRG, and threatened CRM but no association was noted between risk of death and miR-21 expression. Thus, in this study, we showed that miR-21 expression levels before neoadjuvant therapy had the potential to predict response and that patients with miR-21 overexpression exhibited less response to standard CRT dose. This did not, however, translate in a change in survival.

miR-21 is often upregulated in solid tumors influencing cell proliferation, invasion, and apoptosis (18). Considered to be an oncomiR, multiple studies report its role in CRC biology as a screening, diagnostic, and prognostic biomarker (6, 19–23). Also, miR-21 upregulation has been related to advanced stage, presence of positive lymph nodes, venous invasion, and metastatic behavior (24, 25).

In contrast to colonic cancer, very limited data is available on miRNA expression and response to CRT in RC (26–28) with most patients treated with 5-FU-based therapies and not capecitabine. So far, miR-21 has been described to induce resistance to 5-FU when overexpressed in colon cancer cells (13, 29), which could eventually explain its effect regarding 5-FU-based CRT response.

Literature is controversial regarding the use of miR-21 as biomarker of response in RC. In one study with 76 RC

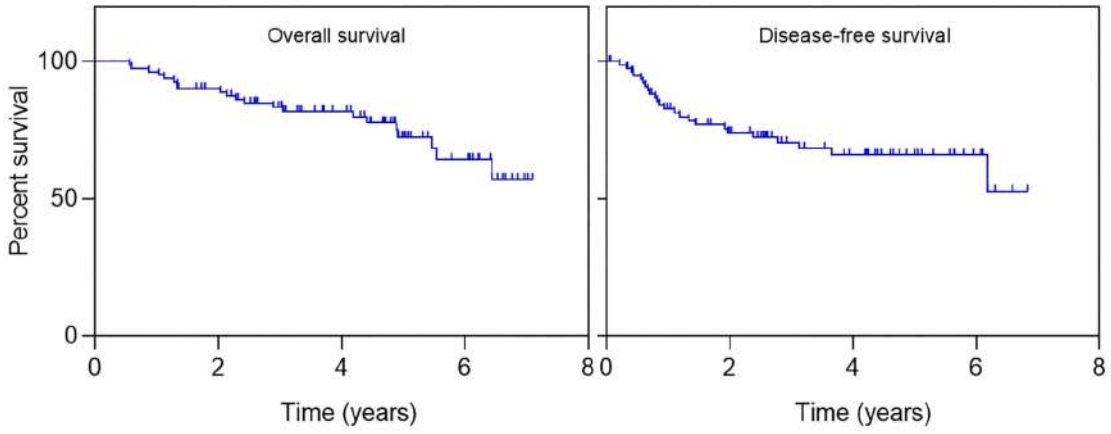

FIGURE 2 | Patient outcomes in miR-21-expressing patients. Kaplan–Meier curves for overall survival and disease-free survival.

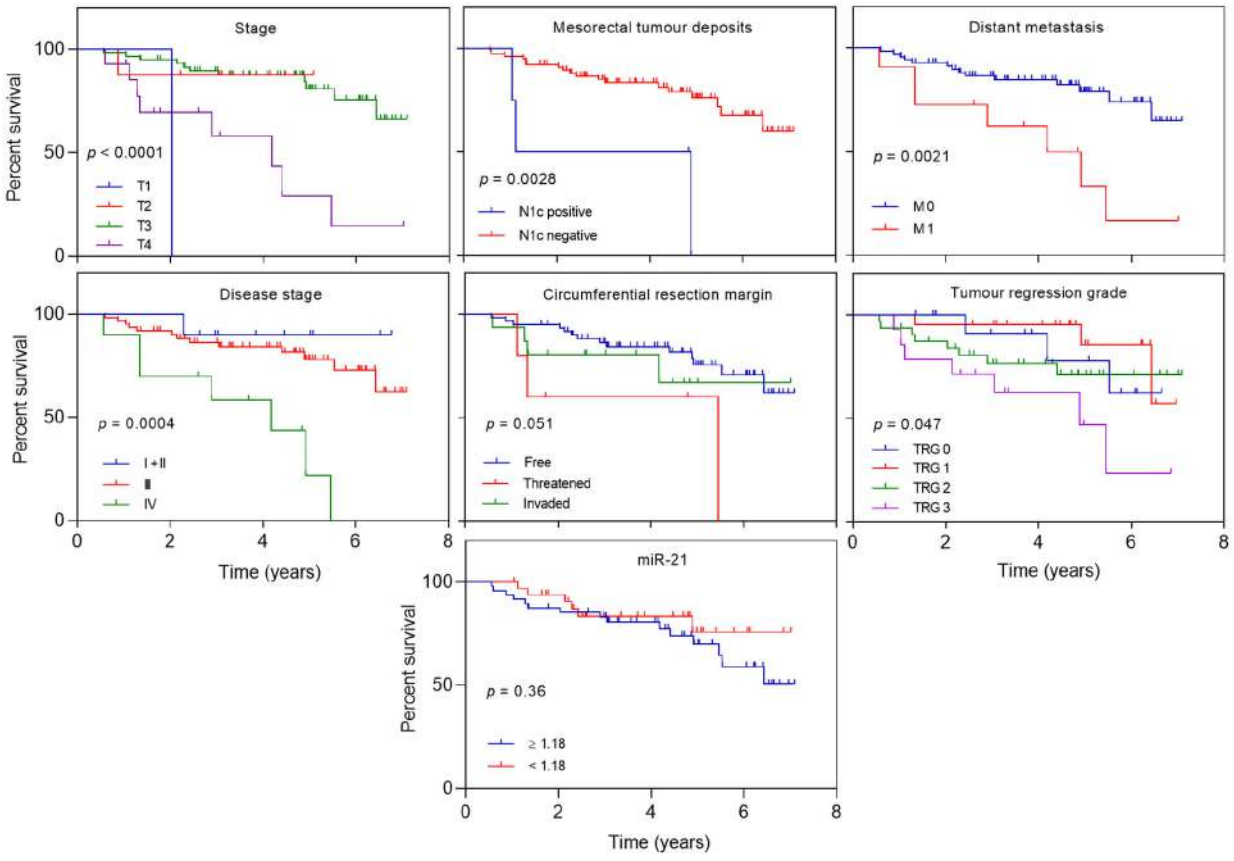

FIGURE 3 | Overall survival according to clinical and oncological parameters. Kaplan–Meier curves estimating overall survival according to stage, mesorectal tumor deposits (cN1c), M, stage, circumferential resection margin (CRM) involvement, tumor regression grade and levels of miR-21.

biopsies, high pre-CRT miR-21 could discriminate responders from non-responders with an OR of 9.75 (95% CI 2.24–42) (30). Recently, 96 complete responders had significantly inferior miR-21 expression comparing with patients with incomplete response ($p = 0.01$), with an AUC of 0.669 (95% CI 0.55–0.79, $p = 0.01$) (31). These observations are in accordance with our own results and with the well-reported miR-21 oncomiR function. Contrarily, in another study, 40 RC patients

TABLE 6 | Patient survival according to miR-21 expression and clinical parameters.

		Patients $n = 82$	Deaths $n = 21$	Survival		Simple cox proportional hazards models			
				Mean	*p*-Value	Coef	HR	95% CI	*p*-Value
miR-21	<1.18	34	6	6.04			1.00		0.36
	≥1.18	48	15	5.50	0.36	0.44	1.56	0.60–4.03	
Age	<60	17	3	5.81	0.58		1.00		0.57
	>60	65	18	5.51		0.35	1.42	0.41–4.8	
Sex	Male	56	16	5.56	0.57		1.00		0.57
	Female	26	5	5.82		−0.29	0.75	0.27–2.04	
Tumor location	1/3 upper	17	3	6.09	0.14		1.00		
	1/3 middle	24	5	6.13		0.05	1.045	0.25–4.40	0.94
	1/3 lower	41	13	5.16		0.91	2.49	0.70–8.85	0.158
ASA score	1 + 2	55	14	5.71	0.97		1.00		
	3 + 4	19	5	5.44		0.10	1.11	0.39–3.094	0.879
	ND	8	2	5.10		0.12	1.12	0.25–4.99	0.986
Stage	I+II	10	1	6.32	0.0004		1.00		
	III	61	13	5.74		0.83	2.31	0.30–17.65	0.4218
	IV	11	7	3.54		2.41	11.10	1.34–91.88	0.0256
Grade	Low	77	19	5.74	0.41		1.00		
	High	5	2	4.87		0.60	1.83	0.42–7.88	0.42
CRM	Free	61	14	5.91	0.051		1.00		
	Threatened	5	3	3.77		1.45	4.24	1.19–15.08	0.025
	Invaded	16	4	5.47		0.51	1.67	0.54–5.142	0.37
EMVI	Negative	77	20	4.45	0.77		1.00		0.768
	Positive	5	1	4.20		0.31	1.36	0.17–10.41	
N1c	Negative	78	18	5.15	0.0028		1.00		0.00788
	Positive	4	3	2.98		1.69	5.47	1.56–19.14	
Extramesorectal nodes	Negative	55	13	5.77	0.26		1.00		
	Positive	27	8	5.15		0.51	1.67	0.68–4.07	0.263
cT	T1-3	68	13	6.05	0.0001		1.00		
	T4	14	8	3.73		1.56	4.78	1.96–11.66	0.0006
cN	0	8	1	6.25	0.42		1.00		
	1	74	20	4.48		0.81	2.24	0.29–16.7	0.432
cM	0	71	14	5.98	0.0021		1.00		
	1	11	7	4.02		1.33	3.78	1.52–9.4	0.00416
TRG	0	15	3	5.94	0.047		1.00		
	1	21	3	6.32		0.49	0.61	0.12–3.05	0.5504
	2	32	8	5.54		0.34	1.41	0.37–5.35	0.6130
	3	14	7	4.31		1.18	3.25	0.83–12.71	0.0897
Chemotherapy	Capecitabine	76	19	5.24	0.47		1.00		
	5-FU	6	2	4.83		0.54	1.71	0.39–7.43	0.476
Post-op complications	Negative	38	9	5.85	0.6		1.00		
	Positive	44	12	5.55		0.23	1.26	0.53–0.98	0.604

Kaplan–Meier estimates, simple cox proportional hazards model. From the initial group of 91 patients, 82 expressed miR-21.
HR, hazard ratios; CI, confidence interval; ASA, American Society of Anaesthesiologists; CEA, carcinoembrionary antigen; CRM, circumferential resection margin; EMVI, extramural vascular invasion; TRG, tumor regression grade.

treated with 5-FU-based CRT had higher miR-21 in post-CRT tumor tissue than in pre-CRT tumor and post-CRT normal tissues (7). It has also been reported overexpression of miR-21 in patients with complete response (32, 33). It is important to note, however, that in one of these studies, the responder group involved a different set of patients, including individuals submitted to surgery with pathological complete response (pCR) and patients with complete clinical response (cCR) not treated with surgery but only observed by follow up (33). The latest might have had undetectable residual disease and not be a real pCR. This different response assessment invalidates an accurate comparison of results and

TABLE 7 | Association between patients survival and miR-21 expression.

		Multiple cox proportional hazards models				Multiple cox proportional hazards models			
		Coef	**HR**	**95% CI**	***p*-Value**	**Coef**	**HR**	**95% CI**	***p*-Value**
miR-21	<1.18			Not included			1.00		
	≥1.18					0.99	2.68	0.86–8.36	0.089
Mesorectal deposits	Negative		1.00				1.00		
	Positive	1.84	6.26	1.74–22.48	0.005	2.49	12.17	2.61–56.70	0.001
cT	T1-3		1.00				1.00		
	T4	1.63	5.09	2.06–12.61	0.0004	1.69	5.45	2.17–13.63	0.0003
C-statistics				0.671				0.674	

Multiple Cox Proportional Hazards Models obtained with stepwise variable selection.
HR, hazard ratios; CI, confidence interval.

may explain the distinct observations when compared with our work.

Overall, the heterogeneity of results is related to the fact that most published studies included patients with colon and RC, 2 distinct entities with different treatment strategies that previous contributions failed to separate. Patient variability, nature of biological samples (blood, tissue, serum, or feces), miRNA extraction, array platforms, bioinformatics analysis, and different TRG grading systems also contribute to these discrepancies. Likewise, it is possible that population may have different miRNA signatures and transcriptome vary according to tumor site.

In this study, we recognized the significance of miR-21 expression in RC in response to neoadjuvant CRT. Although including a sizeable cohort with uniform sampling and treatment, there is a potential for intratumoral heterogeneity and results are currently being validated in a prospective series. If confirmed as a biomarker, translation to clinical practice with miR-21 inclusion in treatment algorithms may allow a stratification of responders and better selection of candidates for CRT.

Of note, in addition to possible markers of response and prognosis at the time of diagnosis, miRNAs may be potential therapeutic targets *via* reintroducing miRNAs absent in carcinogenic pathways or by inhibiting oncomiRs (34–36). Likewise, affecting miRNAs implicated in the mechanism of resistance to CRT may improve the therapeutic outcome. The biggest challenge will continue to be the identification of miRNA targets that shed light on our understanding of downstream cellular mechanisms of resistance to CRT.

In conclusion, the present study suggests miR-21 as a potential biomarker of pathological response in RC. The results provide an association between a miRNA in the neoadjuvant therapy setting and tumor regression with significant implications that strengthen the role of miRNAs as predictors of response. This work further emphasizes the need for prospectively conducted trials of miRNA as biomarkers in RC patients treated with CRT.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institution's Ethical Committee (Comissão de Ética para a Saúde do Hospital Beatriz Ângelo) on 13th March 2017. The study was registered in the Portuguese Data Protection Agency (Comissão Nacional de Protecção de Dados) on 27th January 2017. Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection.

AUTHOR CONTRIBUTIONS

SO: study conception and design, funding, sample collection, sample treatment, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article. CM: miRNA isolation, expression analysis, interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article. SV: statistical analysis of the data and final approval of the article. AC: miRNA isolation, expression analysis, interpretation of the data, and final approval of the article. MF and DA: sample collection and critical revision of the article for important intellectual content. RC and RM: critical revision of the article for important intellectual content and final approval of the article. CR: study design, funding, critical revision of the article for important intellectual content, and final approval of the article. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.577653/full#supplementary-material

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Article

Evaluation of Tissue and Circulating miR-21 as Potential Biomarker of Response to Chemoradiotherapy in Rectal Cancer

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Abstract: Response to chemoradiotherapy (CRT) in patients with locally advanced rectal cancer (RC) is quite variable and it is urgent to find predictive biomarkers of response. We investigated miR-21 as tissue and plasma biomarker of response to CRT in a prospective cohort of RC patients; The expression of miR-21 was analyzed in pre- and post-CRT rectal tissue and plasma in 37 patients with RC. Two groups were defined: Pathological responders (TRG 0, 1 and 2) and non-responders (TRG 3). The association between miR-21, clinical and oncological outcomes was assessed; miR-21 was upregulated in tumor tissue and we found increased odds of overexpression in pre-CRT tumor tissue (OR: 1.63; 95% CI: 0.40–6.63, $p = 0.498$) and pre-CRT plasma (OR: 1.79; 95% CI: 0.45–7.19, $p = 0.414$) of non-responders. The overall recurrence risk increased with miR-21 overexpression in pre-CRT tumor tissue (HR: 2.175, $p = 0.37$); Significantly higher miR-21 expression is observed in tumor tissue comparing with non-neoplastic. Increased odds of non-response is reported in patients expressing higher miR-21, although without statistical significance. This is one of the first studies on circulating miR-21 as a potential biomarker of response to CRT in RC patients.

Keywords: biomarkers; miR-21; chemoradiotherapy; rectal cancer; therapy response; tumor regression grade

1. Introduction

Rectal cancer (RC) is one of the most prevalent cancers in the world [1] but, despite great progress in treatment options, chemoradiotherapy (CRT) is still ministered in the majority of locally advanced cases [2]. After neoadjuvant treatment, almost 30% of patients exhibit resistance to CRT having no benefit from this therapy [3]. In fact, non-responders are at increased risk of disease progression and toxicity related to CRT. Currently, we cannot predict response and the complications associated with this treatment should not be underestimated. There is an urgent need to identify patients that will not benefit from CRT and thus avoid unnecessary morbidity.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs with a post-transcriptional function of inhibiting mRNA translation. These molecules seem to regulate carcinogenic pathways and the potential role in oncogenesis hypothesized their use as biomarkers in cancer diagnostic and prediction

of response to therapy [4]. In fact, miRNAs associated with colorectal cancer (CRC) have been identified in tumor tissue, however, the need for a non-invasive prediction tool prompted their investigation in serum and plasma as circulating markers.

One of the most studied miRNAs is oncomiR-21, demonstrated as a potential diagnostic and prognostic biomarker for CRC, often up-regulated in serum and solid tumors [5–13]. In CRC, miR-21 up-regulation has been related to advanced stage, positive lymph nodes, venous invasion, and metastatic behavior [10–12,14]. Indeed, miR-21 plays a key role in several biological processes needed for tumorigenesis, including resistance to apoptosis, proliferation, evasion to growth suppressors, replicative immortality, and tumor promoting inflammation [15]. miR-21 oncogenic function is exerted mainly through the suppression of a large number of genes that participate directly or indirectly in the extrinsic or intrinsic apoptosis pathways (PDCD4, PTEN, TPM1, MARCKS, HNRPK, TP63, IL12A, JAG1, BTG2, LRRFIP1, BMPR2, TGFBR2, CDC25A, PELI1, ANKRD46, CDK2AP1, MEF2C, MSH2, MSH6, PPARA, RASGRP1, FASLG, TIMP3, ANP32A, SMARCA4, and THRB). In addition, miR-21 is also a negative regulator of p53 signaling and promotes NF-kB, implicated in deregulation of glucose flux and oxidative phosphorylation [15].

However, in rectal cancer (RC) the role of miR-21 as predictor of response to CRT and its association with oncological outcomes has not been fully elucidated. Although one study has demonstrated overexpression of miR-21 in pre-CRT tumor tissue of patients with complete pathological response [16], others have shown that high miR-21 levels associated with worse pathological response, discriminating responders from non-responders [17,18]. Moreover, we have also identified, in a retrospective study, an association between miR-21 expression in pre-CRT rectal tumor tissue and tumor regression grade (TRG), with higher levels correlating with worse pathological response [19]. On the other hand, scarce studies have investigated the potential of circulating miR-21 as a molecular predictor of response in the neoadjuvant therapy setting.

In the present study, using a prospective cohort of patients with RC, we investigated the relation between tissue and plasma miR-21 and evaluated its potential use as a tissue and circulating biomarker of response to CRT. The association between miR-21 and clinical and oncological outcomes was also assessed.

2. Results

2.1. Patient Clinical Parameters

Clinical and demographic features of all 37 patients are summarized in Table 1.

Table 1. Patient clinical parameters.

Clinical Parameters		Patients (*n* = 37)
Gender, *n* (%)	Male	25 (68)
	Female	12 (32)
Age, median		62 (42–88)
BMI, median		25 (20–35)
ASA score, *n* (%)	Not discriminated	3 (8)
	I	0 (0)
	II	22 (60)
	III	12 (32)
	IV	0 (0)
Tumor grade	G1/G2	29 (78)
	G3/G4	2 (6)
	Not discriminated/determinable	6 (16)
Tumor location (%)	1/3 superior	1 (3)
	1/3 medium	14 (38)
	1/3 inferior	22 (59)

Table 1. *Cont.*

Clinical Parameters		Patients (n = 37)
Tumor extension (mm), median		55 (19–90)
Distance to anal verge (mm), median		50 (0–100)
cT	1	0 (0)
	2	7 (19)
	3	25 (68)
	4	5 (13)
cN	0	3 (8)
	+	34 (92)
cM	0	35 (95)
	1	2 (5)
CRM, *n* (%)	Free	17 (46)
	Threatened	4 (11)
	Invaded	16 (43)
EMVI, *n* (%)	Negative	25 (68)
	Present	12 (32)
c Stage, *n* (%)	I	0 (0)
	II	2 (5)
	III	33 (90)
	IV	2 (5)
CEA (mg/mL), median		1.7 (0.5–96)
CRT	5-FU based	4 (11)
	Capecitabine based	33 (90)
TRG (CAP), *n* (%)	0	9 (24)
	1	7 (19)
	2	5 (14)
	3	16 (43)

BMI: Body Mass Index; ASA: American Society of Anesthesiologists; CRM: circumferential resection margin; EMVI: extramural vascular invasion; CEA: carcinoembrinonary antigen; CRT: chemoradiotherapy; MR: magnetic resonance; TRG: tumor regression grade; CAP: College of American Pathologists.

2.2. miR-21 Expression in Responders and Non-Responders

miRNA expression profile was analyzed in non-neoplastic and tumor rectal tissues as well as in plasma, collected before and after CRT. The differences observed when comparing responders (TRG 0-2) and non-responders (TRG 3) are demonstrated in Figure 1. In responders, miR-21 revealed significantly higher expression (p = 0.0013) in pre-CRT tumor tissue when compared with non-neoplastic tissue. The same expression profile was observed in post-CRT tissue samples with higher levels of miR-21 in the tumor tissue. However, this profile was also detected in non-responders with overexpression of miR-21 detected in pre-CRT (p = 0.0004) and post-CRT tumor tissue when compared with non-neoplastic tissue (Figure 1A).

Regarding miR-21 expression analysis in plasma (Figure 1B), a slight increase with no statistical significance was observed in post-CRT plasma miR-21 expression in responders comparing with pre-CRT samples. Again, no differences were evident before and after treatment in non-responders.

2.3. Clinical Parameters and TRG

There was no statistically significant association between clinical parameters and TRG (Table 2). Nevertheless, we observed in our sample a reduced odds of non-response (TGR 3) in women (OR: 0.54; CI: 0.13–2.27; p = 0.4), individuals older than 60 years (OR: 0.39; CI: 0.09–1.74; p = 0.217), ASA 3 (OR: 0.8; CI: 0.21–3.03; p = 0.746), in patients treated with capecitabine based CRT when compared to 5-FU (OR: 0.34; CI: 0.03–4.32; p = 0.390) and tumors located in the inferior 1/3 of the rectum (OR: 0.79; CI: 0.21–2.97; p = 0.73). On the other hand, the odds of non-response were 6 times higher for cT3 and T4 when compared to cT1 or cT2 (OR: 6.0; CI: 0.64–56.06, p = 0.09).

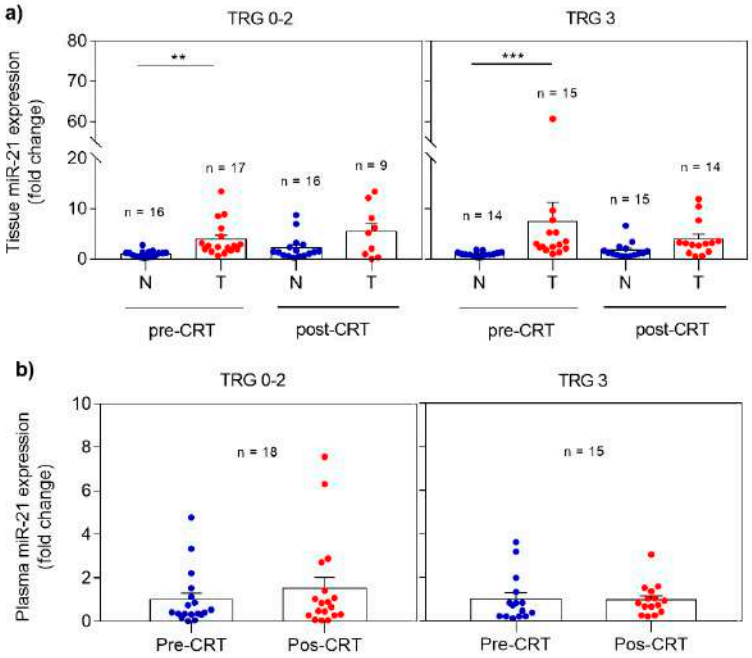

Figure 1. Expression profile of miR-21 in pre- and post-CRT samples in responders (TRG 0-2) and non-responders (TRG 3). (**a**) miR-21 levels in non-neoplastic and tumor tissues; (**b**) miR-21 levels in plasma. Fold changes in tissue and plasma miR-21 expression are calculated from pre-CRT non-neoplastic tissue and pre-CRT plasma expression, respectively. Data are mean ± SEM. N corresponds to non-neoplastic tissue and T to tumor tissue. ** $p \leq 0.01$, *** $p \leq 0.001$.

Table 2. Clinical parameters and TRG.

Simple Logistic Regression		OR	95% CI	*p* Value
Continuous Variables				
BMI		1.029	0.2649–3.993	0.968
Age		0.392	0.0887–1.735	0.217
Categorical Variables				
Gender	Female Male	0.542	0.1291–2.272	0.406
Tumor Location	Superior 1/3 Medium 1/3 Inferior 1/3	0.791	0.2107–2.972	0.732
ASA	1 + 2 3	0.800	0.2114–3.028	0.746
CRM MR	Free Threatened, invaded	1.169	0.3162–4.320	0.817
Extramesorectal nodes	Negative Positive	0.542	0.1291–2.272	0.406
cT	T1-2 T3-4	6.000	0.6421–56.062	0.090
cN	0 +	0.350	0.0289–4.246	0.399
cM	0 1	1.333	0.0770–23.085	0.845
Chemotherapy	Capecitabine 5-FU	0.342	0.0280–4.320	0.390

Simple logistic regression analysis using TRG as dependent variable (TRG 3) and clinical/ molecular variables as independent variables. OR: odds ratio of non-response (TRG 3); TRG: Tumor regression grade; CI: confidence interval; BMI: body mass index; ASA: American Society of Anesthesiologists; CRM: circumferential resection margin; MR: magnetic resonance.

2.4. miR-21 Expression and TRG

To study a possible association between miR-21 expression and TRG, we resorted to ROC curve analysis to determine the optimal cut-off that maximized sensitivity, specificity and distinction between responders and non-responders (Table S1). We found increased odds of non-response in patients with higher miR-21 expression (>1.2) in pre-CRT non-neoplastic rectal tissue (OR: 1.2; CI: 0.24–6.06, $p = 0.828$) and in patients with levels higher than 2.61 in pre-CRT tumor tissue (OR: 1.6; CI: 0.40–6.63, $p = 0.49$) (Table 3).

Table 3. miR-21 expression and TRG.

Variables		OR	95% CI	*p* Value
miR-21 pre-CRT non-neoplastic	≤1.2			
	>1.2	1.20	0.237–6.064	0.828
miR-21 pre-CRT tumor	≤2.61			
	>2.61	1.63	0.402–6.625	0.498
miR-21 pre-CRT plasma	≤0.54			
	>0.54	1.20	0.237–6.064	0.828
miR-21 post-CRT plasma	≤0.84			
	>0.84	1.09	0.276–4.330	0.900

Simple logistic regression according to cut-offs determined with ROC curve analysis. OR: odds ratio of non-response (TRG 3); CI: confidence interval.

Regarding plasmatic miR-21, there was also an increased odds of TRG 3 in patients with pre-CRT miR-21 expression higher than 0.54 (OR: 1.2; CI: 0.24–6.06, $p = 0.828$) and in patients with post-CRT miR-21 levels >0.84 (OR: 1.09; CI: 0.28–4.33, $p = 0.9$) (Table 3).

Overall, in our sample, patients with higher levels of miR-21 in pre-CRT tissue and plasma had less response to CRT.

2.5. Clinical Parameters and miR-21 Expression in Pre-CRT Tumor Tissue and Plasma

In pre-CRT tumor tissue an increased odds of miR-21 overexpression (>2.61 fold change) was observed in patients with cT3-4 (OR: 2.71; 95% CI: 0.44–16.68, $p = 0.28$), TRG 3 (OR: 1.63; 95% CI: 0.40–6.63, $p = 0.498$), local (OR: 1.14; 95% CI: 0.07–20.02, $p = 0.928$) and distant recurrence (OR: 2.73; 95% CI: 0.42–17.65, $p = 0.289$). On the contrary, high miR-21 levels were less likely for subjects older than 60 years (OR: 0.83; 95% CI: 0.19–3.72, $p = 0.81$), obese (OR: 0.38; 95% CI: 0.08–1.69, $p = 0.21$) and ASA 3 (OR: 0.41; 95% CI: 0.09–1.81, $p = 0.24$) (Table 4).

Regarding pre-CRT circulating miR-21, there was an increased probability of miR-21 overexpression (>0.54 fold change) in patients with TRG 3 (OR: 1.79; 95% CI: 0.45–7.19, $p = 0.414$), N+ (OR: 1.75; 95% CI: 0.14–21.44, $p = 0.663$) and distant metastasis (OR: 2.21; 95% CI: 0.07–21.22, $p = 0.896$). However, overexpression was less likely in obese patients (OR: 0.89; 95% CI: 0.22–3.66, $p = 0.87$), cT3 and cT4 (OR: 0.80; 95% CI: 0.14–4.70, $p = 0.80$) and in the presence of distant recurrence (OR: 0.30; 95% CI: 0.07–2.45, $p = 0.32$) (Table 5). Again, overall, patients with miR-21 overexpression in pre-CRT tumor tissue and in blood had less response to CRT.

Table 4. Clinical parameters and miR-21 expression in pre-CRT tumor tissue.

Variables		OR	95% CI	*p* Value
Age	<60 ≥60	0.83	0.19–3.72	0.814
Sex	Male Female	2.1	0.49–8.99	0.322
BMI	Low weight + normal Pre-obesity + obesity	0.38	0.08–1.69	0.206
ASA score	2 3	0.41	0.09–1.81	0.242
Stage pre-CRT	I + II III + IV	0.88	0.57–27.24	0.203
cT	T1 T3 + 4	2.71	0.44–16.68	0.280
cN	0 1	0.87	0.05–15.33	0.928
pTRG	TRG 0 + 1 + 2 TRG 3	1.63	0.40–6.63	0.498
Distant recurrence	No Yes	2.73	0.42–17.65	0.289
Local recurrence	No Yes	1.14	0.07–20.02	0.928

Simple logistic regression analysis using miR-21 expression (> 2.61-fold change) as dependent variable and clinical variables as independent variables. OR of miR-21 > 2.61-fold change. OR: odds ratio; CI: confidence interval; ASA: American Society of Anesthesiologists; BMI: body mass index; CRT: chemoradiotherapy; pTRG: pathological tumor regression grade.

Table 5. Clinical parameters and miR-21 expression in pre-CRT plasma.

Variables		OR	95% CI	*p* Value
Age	<60 ≥60	4.14	0.71–24.16	0.106
Sex	Male Female	1.73	0.40–7.46	0.465
BMI	Low weight + normal Pre-obesity + obesity	0.89	0.22–3.66	0.873
ASA score	2 3	1.75	0.43–7.17	0.442
Stage pre-CRT	I + II III + IV	0.82	0.05–14.39	0.896
cT	T1 + T2 T3 + T4	0.80	0.14–4.70	0.808
cN	N0 N1	1.75	0.14–21.44	0.663
cM	M0 M1	2.21	0.07–21.22	0.896
pTRG	TRG 0 + 1 + 2 TRG 3	1.79	0.45–7.19	0.414
Distant recurrence	No Yes	0.40	0.07–2.45	0.320

Simple logistic regression analysis using miR-21 expression (>0.54-fold change) as dependent variable and clinical variables as independent variables. OR of miR-21 > 0.54. OR: odds ratio; CI: confidence interval; ASA: American Society of Anesthesiologists; BMI: body mass index; CRT: chemoradiotherapy; pTRG: pathological tumor regression grade.

2.6. miR-21 Expression and Oncological Outcomes

With a median follow up of 603 (196–1007) days, we report 3 (8%) mortality cases, 2 (5%) cases of local recurrence (LR) and 7 (19%) of distant recurrence (DR). The low number of death cases precluded correct estimation of overall survival (OS) but 3 and 5-year predicted disease free survival (DFS) were 67 and 46%, respectively (Figure 2).

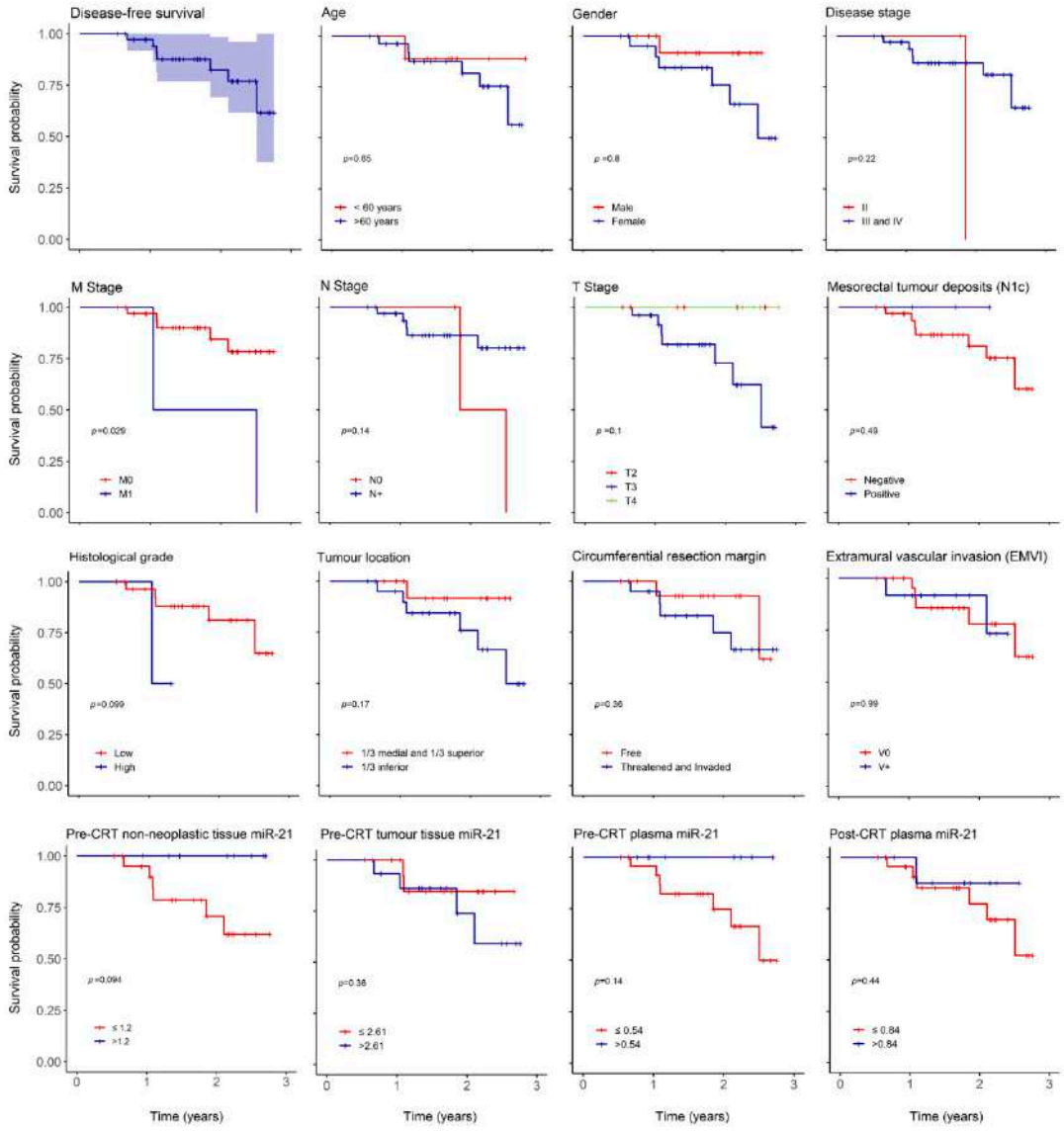

Figure 2. Overall disease-free survival (DFS) and according to clinical and oncological parameters. Kaplan–Meier curves estimating 3-year overall DFS in patients expressing miR-21 and according to age, gender, disease stage, M stage, N stage, T stage, mesorectal tumor deposits (N1c), histological grade, tumor location, circumferential resection margin, extramural vascular invasion (EMVI), pre-CRT non-neoplastic tissue miR-21, pre-CRT tumor tissue miR-21, pre-CRT plasma miR-21 and post-CRT plasma miR-21.

The overall recurrence hazard risk (HR) increased in women (HR: 1.218, $p = 0.797$), older patients (HR: 1.64, $p = 0.65$), lower tumor location (HR: 4.03, $p = 0.19$), threatened or invaded circumferential resection margin (CRM) (HR: 2.14, $p = 0.37$) and TRG 3 (HR: 3.95, $p = 0.11$) (Table 6). Overall recurrence

HR also augmented in individuals with higher pre-CRT tumor tissue miR-21 expression (HR 2.175, $p = 0.37$) (Table 6).

Table 6. Clinical parameters, miR-21 levels, and overall recurrence.

Variables		Total	DFS	r Mean	Simple Cox Proportional Hazard Model	
					HR	*p* Value
Tumor location	Superior + medium Inferior	15 22	1 6	2.53 2.25	4.027	0.199
Age	<60 ≥60	10 27	1 6	2.54 2.38	1.637	0.651
Gender	Male Female	25 12	4 3	2.41 2.39	1.218	0.797
CRM	Free Threatened/invaded	17 20	2 5	2.53 2.30	2.135	0.368
TRG	0–2 3	21 16	2 5	2.57 2.21	3.950	0.108
miR-21 pre-CRT tumor	≤2.61 >2.61	17 15	2 4	2.47 2.26	2.175	0.37
miR-21 pre-CRT plasma	≤0.54 >0.54	18 15	5 2	2.27 2.45	0.464	0.36

Simple Cox Proportional Hazards Model using global recurrence as dependent variable and clinical parameters as independent variables. HR: hazard ratio; CRM: circumferential resection margin; TRG: tumor regression grade; DFS: disease free survival.

As expected, there was an impact in 3-year DFS in relation to histological grade ($p = 0.09$) and distant metastasis ($p = 0.029$) (Figure 2) but no influence was noted in age, gender, T or N stage, tumor location, threatened or invaded CRM, N1c or EMVI. There was also a decrease in 3-year DFS in patients with higher pre-CRT tumor miR-21 ($p = 0.36$) and in patients with lower miR-21 in pre-CRT non-neoplastic tissue ($p = 0.09$) and plasma ($p = 0.14$).

We also evaluated the correlation between pre- and post-CRT circulating and tissue miR-21. Results showed, however, very week correlations (Figure S1). There was a positive but frail correlation between pre-CRT plasma and tumor miR-21 with an increase in tissue miR-21 with escalation expression in blood (r = 0.002, $p = 0.993$).

3. Discussion

The interest in identifying biomarkers for cancer has led both researchers and clinicians to focus on miRNAs [20]. Some studies have investigated the diagnostic and prognostic value of miR-21 in RC as well as its potential to predict response to CRT [16–18]. However, the conclusions obtained from these studies were inconsistent granting the need to further explore the clinical significance of miR-21 as a biomarker in this setting. Generally, findings associate a superior miR-21 expression with a non-or incomplete response. In fact, in a previous retrospective study, our group also identified an association between miR-21 overexpression in pre-CRT rectal tumor tissue and worse pathological response [19]. In that study, this miRNA could differentiate incomplete from complete responders and potentially be used as biomarker to predict TRG. Nevertheless, the evaluation of circulating miR-21 as a non-invasive biomarker of response to CRT in rectal cancer has never been investigated.

The first detection of miRNAs in body fluids occurred when miR-21 was found in the serum of B-cell lymphoma patients [21]. Since then, up-regulated miR-21 levels in plasma have been associated with solid cancers (glioblastoma, breast cancer, and pancreatic cancer) [22] and therefore it was termed oncomiR.

Levels of miRNAs in plasma are remarkably stable, reproducible, consistent among individuals of the same species [23] and cells actively release the majority of circulating miRNAs. The idea of a correlation between circulating and tissue miRNA supports the hypothesis that plasmatic miRNAs can serve as biomarkers of disease or disease response. miRNAs appear to demonstrate the same change

in expression, either increased or decreased, in plasma or serum and tumor tissues of patients with various types of cancer [23]. However, only few studies focused on the detection of circulating miRNAs in CRC patients [24–26] and this could be attributed to challenges in plasma miRNA extraction and lack of consensus about internal controls for qRT-PCR and normalization.

Clinical significance of circulating miR-21 levels in CRC remains, in fact, not fully understood. Some studies report on seric miR-21 as a discriminative biomarker of colorectal neoplasms from healthy controls [9,27–37] and from benign or premalignant adenoma [33,38]. Circulating miR-21 has also been correlated with tumor size, grade of differentiation, invasion, metastasis [32], recurrence, and survival [6]. The expression of miR-21 has been found significantly increased in preoperative serum from CRC patients who did not received neoadjuvant therapy and this correlated with tumor size, poor survival, and lymph node metastasis [14,37]. Another important issue is that, in reality, very few studies differentiate between colon and rectal cancer patients and these are two different entities with distinct treatment options. In fact, serum miR-21 levels seem to be upregulated in rectum cancer tissue in comparison to colon cancer [39].

In the present work, we aimed to investigate the potential of tissue miR-21 as a biomarker of response to CRT in a prospective cohort of RC patients and validate our previous retrospective results as well as assess circulating miR-21 in this setting. Although we could not demonstrate the efficacy of tissue and plasma miR-21 to differentiate responders (TRG 0-2) from non-responders (TRG 3), we did find an odds increase of non-response in all patients expressing higher miR-21 levels. miR-21 was upregulated in tumor tissue and there was an increased probability of pre-CRT tumor tissue miR-21 overexpression in patients with non-response. In addition, in this study overall recurrence hazard risk increased in patients with less response, threatened or invaded CRM, and higher pre-CRT tumor tissue miR-21 levels. Regarding 3-year DFS analysis, we observed a decrease in survival in patients with higher miR-21 levels in pre-CRT tumor tissue, while overexpression of miR-21 was related to a better survival in pre-CRT non-neoplastic tissue. This is concordant with our hypothesis that when comparing pre-CRT non-neoplastic and tumor tissue we predict response to treatment, where higher miR-21 in pre-CRT tumor tissue in comparison with non-neoplastic tissue is indicative of a worse response to treatment, whereas higher miR-21 in pre-CRT non-neoplastic tissue is associated with better response to CRT. Considering plasma miR-21 analysis, although with no statistical significance, we observed increased odds of pre-CRT circulating miR-21 overexpression in non-responders (TRG 3). Overall, these results are in line with our retrospective study that found a significant association of miR-21 overexpression in pre-CRT rectal cancer tissue with worse response to neoadjuvant therapy [19]. Moreover, pre-CRT plasmatic miR-21 may be also related to less response. To our knowledge, this is one of the first reports in which circulating miR-21 has been investigated as a predictive biomarker of response to neoadjuvant CRT in rectal cancer.

Recently, it was observed that circulating exosomal miR-21 could distinguish chemotherapy resistant from chemosensitive CRC patients [40]. This miRNA was shown to be upregulated in the exossomes of chemoresistant CRC cell lines and in pre-chemotherapy exosomal serum of patients that did not respond to treatment. These results are according to our suggestion that overexpression of pre-CRT circulating miR-21 may be indicative of worse response to CRT in rectal cancer setting, possibly related to the chemotherapy effect. Interestingly, in the present study we also observed a reduced odd of non-response in patients treated with capecitabine based CRT when compared to 5-FU (OR: 0.34; CI: 0.03–4.32; $p = 0.390$). In contrast to 5-FU-based therapies, very limited data is available on miRNA expression and response to CRT with capecitabine. Nevertheless, this outcome lines up with our retrospective study, where 5-FU-treated patients also presented reduced odds of incomplete response (OR: 0.19; 95% CI: 0.03–1.12, $p = 0.05$).

The differences observed between the current work and our previous report, that showed the potential of miR-21 as a discriminative biomarker of response to CRT, are probably due to the limitation in sample size in this prospective study as well as the different TRG based definition of patient groups. Besides, although this group of patients includes uniform sampling and treatment, there is a potential

for intratumoral heterogeneity and thus, validation of our results in a larger cohort still needs to be performed.

4. Materials and Methods

This was a prospective observational study. Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institutional Human Research Committee and Ethical Committee (Hospital Beatriz Ângelo; 13 March 2017, Project Identification Number 0240). The study was registered in the Portuguese Data Protection Agency.

4.1. Patients and Tissue Samples

A total of 37 patients diagnosed with RC (stage I-IV, American Joint Committee on Cancer, AJCC) between April 2017 and June 2019 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal) treated with long course CRT and proctectomy were eligible. Patients had a preoperative staging with pelvic magnetic resonance (MR), thoraco-abdomino-pelvic computed tomography (CT) and endoanal ultrasound when pelvic MR was not clinically possible. Histopathological features were confirmed by pathological analysis and patients were staged according to TNM staging system (8th edition, 2017). Patients with other histological types of rectal malignancy, not submitted to CRT or surgical resection, pregnant or under the age of 18 were excluded.

Two groups of patients were defined: responders (TRG 0, 1, and 2) composed of a total of 21 patients and non-responders (TRG 3) composed of a total of 16 patients.

Fresh frozen tissue samples were collected before and after CRT, during pre-therapeutic colonoscopy and from the protectomy specimen, respectively. Pre-CRT rectal tumor biopsies were gathered from all patients but post-CRT tumor tissues were available only from patients without a pathological complete response. To allow a direct comparison of rectal cancer to matched non-neoplastic rectal mucosa, we collected corresponding adjacent (>1 cm distant) non-tumor tissue both in biopsies and protectomy specimens. Retrieved tumor and non-neoplastic tissue underwent histological confirmation by a pathologist. A fixed amount of tissue (80 μm) was extracted across samples, immediately frozen with CO_2 prior to storage at −80 °C. In addition, liquid biopsies (plasma) were also collected from 33 patients, before and after CRT, at the time of pre-treatment staging colonoscopy and 24 h after proctectomy. Peripheral blood was collected in vacutainer liquid EDTA 6-mL blood collection tubes and peripheral blood cells and plasma were separated by density gradient separation. Plasma was then stored and frozen at −80 °C until RNA extraction.

4.2. Neoadjuvant Treatment

All patients underwent neoadjuvant CRT that consisted of a total dose of 50.4 Gy of pelvic irradiation, 5 times a week, with a daily fraction of 2 Gy using at least a four-field technique. Radiation was delivered with capecitabine (825 mg/m^2/day) or 5-fluoruocil (5-FU) (1000 mg/m^2/ day on day 1 to 5 and days 29 to 33). Surgery was performed 10–12 weeks after CRT.

4.3. Assessment of Pathological Response

Pathology specimens were graded by Tumor Regression Grade (TRG) according to the College of American Pathologists guidelines (CAP, TNM 7th edition). Two independent pathologists blinded to patient clinical data evaluated TRG categorizing tumors in: TRG 0 or complete response (no viable tumor cells), TRG 1 or moderate score (single cells or little groups of cancer cells), TRG 2 or minimal response (residual cancer outgrown by fibrosis), TRG 3 or poor response (minimal or no tumor killing with extensive residual cancer).

4.4. Follow up

Patients had a median of 603 (196–1007) days of follow up with no patients lost.

4.5. RNA Isolation from Fresh Frozen Tissues and Serum

Total RNA was extracted using Ribozol™ reagent (VWR International, Radnor, PA, USA) in pre- and post-CRT fresh frozen non-neoplastic and tumor rectal tissues samples according to the manufacturer's instructions, whereas miRNeasy serum/plasma advanced kit (Qiagen, GmbH, Germany) was used to isolate RNA in pre- and post-CRT plasma samples from a total amount of 200 µL of plasma. In plasmatic RNA isolation, an exogenous control was added to each sample to monitor extraction efficiency and to further normalize miRNA expression data. Thus, $1.6x10^8$ copies/µL of synthetic spike-in control *Caenorhabditis elegans* miR-39 5′-phosphorylated (cel-miR-39-3p_5P) was added according to the miRNeasy kit instructions. RNA extracted from tissue and serum was eluted in 50 µL and 20 µL of RNase-free water, respectively. For a better evaluation of miRNAs quantity in total RNA, the concentration of miRNA was determined using Qubit™ miRNA Assay kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). All RNA samples were stored at −80 °C.

4.6. cDNA Synthesis and Real-Time PCR (RT-PCR)

cDNA synthesis was performed using TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 2 µL of total RNA (corresponding to 2 ng of RNA extracted from tissue) were extended by a 3′ poly-A tailing reaction and a 5′ adaptor ligation to the mature miRNAs. miRNAs were reverse transcribed into cDNA by reverse transcription using Universal RT primers. In order to improve detection of low-expressing miRNA targets, a pre amplification of the cDNA was performed using the Universal miR-Amp Primers and miR-Amp Master Mix to uniformly increase the amount of cDNA for each target, maintaining the relative differential expression levels. cDNA samples were stored at -20°C. Real-Time PCR was performed on a Quantstudio™ 7 Flex real-time PCR instrument (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) with TaqMan™ Advanced microRNA Assays (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) to assess the expression profile of hsa-miR-21-5p (Assay ID 477975_mir). All reactions were performed in duplicate.

Since a consensual endogenous control for miRNA expression in rectal tissue has still not been determined, normalization was performed with hsa-miR-484 (Assay ID 478308_mir) for tissue miRNA expression analysis. In our previous retrospective study miR-484 was identified as the most stably expressed miRNA with the lowest expression variability when compared with mir-1228-5p, miR-345-5p, miR-103a-3p and the small nuclear (snRNA) U6 and RNU6B, considered endogenous controls for CRC tissues and/or serum. For serum miRNA expression analysis, normalization was performed with cel-mir-39-3p (Assay ID 478293_mir). Expression levels were calculated by the threshold cycle ($2^{-\Delta\Delta Ct}$ method), when amplification values were detected. Due to lack of amplification values of miRNAs detected for all tissues, a variable number of samples have been included in each tissue miRNA expression profile. To determine fold change, pre-CRT non-neoplastic tissue and pre-CRT plasma samples were used as controls in tissue and plasma expression analysis, respectively. Fold change values were calculated as the ratio between miR-21 levels in tissue or plasma and the mean of the controls' values.

4.7. Statistical Analysis

miRNA expression was analyzed using the Graph Pad Prism software package, version 7.0 (GraphPad software Inc., San Diego, CA, USA). Normal distribution was determined using the D'Agostino & Pearson omnibus test. Statistical differences between patient groups in plasma expression data were evaluated by two-tailed non-parametric Mann–Whitney *U* test, whereas tissue expression data was analyzed using the one-way analysis of variance (ANOVA) Kruskal–Wallis non-parametric

Dunn's multiple comparison test. Spearman correlation coefficient was used to test the correlation between plasma and tissue miRNA expression levels. Using contingency tables odds ratio (OR) were estimated and the *p*-value associated were obtained resorting to Fisher test. Receiver operating characteristic curve (ROC) were used to calculate optimal cut-offs for miR-21 in pre-CRT normal, tumor tissue and blood determined as the point closest to the top left part of the plot with perfect sensibility and sensitivity. miR-21 was then dichotomized according to these cut-offs. Kaplan–Meier survival curves were compared with Log-rank test and simple Cox proportional hazards models were adjusted to analyze the association of each variable with disease free survival. Overall survival was not possible to determine in this study due to the reduced number of deaths observed ($n_{\text{death}} = 3$). Data was analyzed with SPSS (IBM, version 20) and R (version 3.0.2). $p \leq 0.05$ acknowledged statistical significance. There was professional statistical review performed in this manuscript.

5. Conclusions

There is an urgent need for biomarkers of response to CRT. In this study, although the efficacy of tissue and plasma miR-21 to differentiate responders from non-responders could not be demonstrated, the odds of non-response in patients overexpressing miR-21 was increased, however, with no statistical significance. The role of miR-21 as a predictive tool for pathological response in RC patients treated with CRT needs to be established in larger cohorts. Confirmation as such would translate into clinical application through inclusion in algorithms of treatment decision, allowing a better selection of candidates for CRT.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-8247/13/9/246/s1, Figure S1: Correlation between pre- and post-CRT miR-21 expression in plasma and tumor tissue, Table S1: Predictive value of miR-21 cut-off.

Author Contributions: S.O., study conception and design, funding acquisition, sample collection, formal analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article; C.M., miRNA isolation, formal analysis, interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article; M.P.F., sample collection, critical revision of the article for important intellectual content; D.A., sample collection, critical revision of the article for important intellectual content; final approval of the article; A.C., miRNA isolation, expression analysis, interpretation of the data, final approval of the article; R.E.C., critical revision of the article for important intellectual content, final approval of the article; R.M., critical revision of the article for important intellectual content, final approval of the article; C.M.P.R., study design, funding acquisition, critical revision of the article for important intellectual content, final approval of the article. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Transanal total mesorectal excision: 3-year oncological outcomes

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Abstract

Background Rectal cancer treatment has evolved with the implementation of new surgical techniques. Transanal total mesorectal excision (TaTME) is the most recent approach developed to facilitate pelvic dissection of mid- and distal rectal tumours. The purpose of this study was to analyse the short- and mid-term oncological outcomes of TaTME.

Methods A study was conducted on patients treated with TaTME for rectal cancer at two colorectal units in Portugal between March 2016 and December 2018. Clinical, pathological and oncological data were retrospectively analysed. Primary endpoints were 3-year overall survival, disease-free survival and local recurrence. Secondary endpoints were clinical and pathological outcomes.

Results Fifty patients (31 males, [62%], median age 66 years [range 40–85 years]) underwent TaTME, 49 (98%) for malignant and 1 (2%) for benign disease. There were no cases of conversion, 49 (98%) patients had complete or near-complete mesorectum, all the resections were R0 with adequate distal and circumferential margins. With a median follow-up of 36 months, there were 2 cases (4%) of local recurrence and 3-year estimated overall survival and disease-free survival were 90% and 79%, respectively.

Conclusions TaTME can provide safe mid-term oncological outcomes, similar to what has been published for classic and laparoscopic TME. Our results also show how demanding this novel approach can be and the consequent need for audited data and standardized implementation.

Keywords Rectal cancer · TaTME · Oncological outcomes

Introduction

Rectal cancer is one of the most prevalent malignancies worldwide and the gold standard treatment is total mesorectal excision (TME). As circumferential, distal margins and mesorectal integrity are the most important prognostic factors for local recurrence (LR), good quality surgery is essential [1]. Risks factors for positive circumferential resection margins and intraoperative technical difficulty are male sex, high body mass index, narrow pelvis, distally located and advanced T-stage lesions [2–4]. On the one hand, optimal surgical outcomes must be guaranteed, on the other TME can be technically challenging due to the difficulty of working in a restricted space with limited vision.

Transanal total mesorectal excision (TaTME) was developed in 2010 to overcome difficulties in the dissection of the pelvis allowing a better visualization of the recto-prostatic/recto-vaginal septum and neurovascular bundles, with improved distal margin definition and avoidance of stapling [5–7]. The use of 2 teams working synchronously decreases surgical time and surgeon fatigue in the critical moment of the surgery. TaTME has, however, introduced new complications, not commonly associated with the classic and laparoscopic approaches, mainly urethral injuries and carbon dioxide embolism [8, 9].

Although it seems that this technique improves short-term clinical outcomes, there are still inconsistencies regarding oncological outcomes. The aim of this study was to investigate mid-term clinical and oncological outcomes of the introduction of TaTME and show the outcome of the

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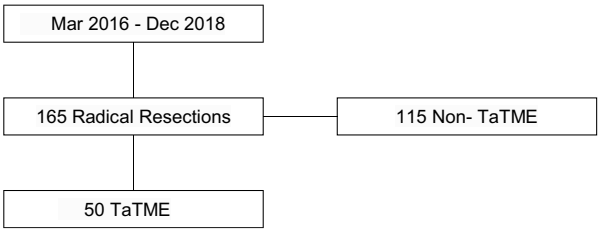

Fig. 1 Case volume of radical resections for rectal cancer in both units during the study period. *TaTME* transanal total mesorectal excision

learning curve, which is accepted to be at least 20 cases per surgeon [10].

Materials and methods

This was a retrospective study of prospectively analysed data. The first 50 consecutive patients with rectal cancer stage I–IV, American Joint Committee on Cancer (AJCC) submitted to TaTME between March 2016 and December 2018 in Hospital Beatriz Angelo and Hospital da Luz, Lisbon were eligible for this study. Our unit's volume of rectal radical resection during the elected study period is shown (Fig. 1). Initially, all patients with rectal cancer (less than 15 cm from the anal verge) were considered elective for TaTME but, due to the possibility of performing the unneeded too distal anastomosis, we changed the selection to patients with cancers of the mid and lower rectum (defined as less than 10 cm and 6 cm from the anal verge, respectively, through rigid sigmoidoscopy and magnetic resonance). All patients accepted this technique through informed consent.

Before starting TaTME technique, surgeons underwent didactic learning, observation of live TaTME procedures and hands-on courses.

Data was gathered from the electronic hospital databases. Primary endpoints were 3-year overall survival (OS), disease-free survival (DFS) and LR. Secondary endpoints were clinical and pathological outcomes.

Statistical analysis

This was a descriptive study and no test was applied. Survival analysis was performed through Kaplan–Meier statistics. SPSS (IBM, version 20) and R (version 3.0.2) were used. $P \leq 0.05$ was considered to be statistically significant. There were no missing data and no patients were lost to follow-up.

Results

Clinical parameters

During the study period, a total of 50 patients had TaTME, (31 [62%] males, median age 66 years [range 40–85 years], median body mass index 26 kg/m^2 [range 19–39 kg/m^2]). Forty-eight (96%) patients had a Eastern Cooperative Oncology Group (ECOG) performance status score of 0, 35 (70%) patients were classified as American Society of Anaesthesiologists (ASA) class II and 7 (14%) had the previous laparotomy for other causes (Table 1).

Preoperative staging and neoadjuvant therapy

Of the 50 patients in the study, 49 (98%) were treated for rectal cancer and 1 (2%) for benign disease (endoscopically non-resectable tubulovilous adenoma with high-grade dysplasia). The neoplasia was localized mainly in the mid- and low rectum with a median distance to the anal verge of 70 mm (range 20–120 mm). All patients underwent preoperative staging with pelvic magnetic resonance imaging (MRI) and computed tomography (CT) scan of the chest, abdomen and pelvis except for 2 that underwent endoanal ultrasound (EAUS) due to metallic prostheses. Pelvic MRI showed mesorectal fascia invaded or threatened in 10 (20%) patients and extramural vascular invasion (EMVI) in 5 (10%). The median level of carcinoembryonic antigen was 1.35 ng/mL (range 1.3–1.4 ng/mL). 24 (48%) patients had neoadjuvant chemoradiotherapy (CRT), 23 with a long-course (LCCRT) and 1 with a short-course (SCRT) regimen. Restaging pelvic MRI was done at 6 weeks post neoadjuvant CRT and 11

Table 1 Clinical parameters

Clinical parameters		Patients ($n = 50$)
Sex, *n* (%)	Male	31 (62)
	Female	19 (38)
Age, years, median (range)		66 (40–85)
BMI, kg/m^2 median (range)		26 (19–39)
PS (ECOG), *n* (%)	0	48 (96)
	1; 2	2 (4)
ASA score, *n* (%)	II	35 (70)
	III	15 (30)
Previous abdominal surgery, *n* (%)	Hysterectomy	2 (4)
	Colectomy	2 (4)
	Appendectomy	2 (4)
	Anterior resection	1 (2)

BMI body mass index, *PS* performance status, *ECOG* Eastern Cooperative Oncology Group, *ASA* American Society of Anaesthesiologists

(46%) patients showed a good response, tumour regression grade 1 or 2 [11] (Table 2).

Surgical technique

All 50 patients had preoperative mechanical bowel preparation and underwent the surgical procedure at a median of 12 weeks (range 7–22 weeks) after CRT. All the procedures were done with a synchronous 2-team approach, transabdominal and transanal, by the same surgical teams. There was no intraoperative mortality (Table 3).

Abdominal approach

The abdominal approach was performed through laparoscopy in 42 (84%) patients and robotically assisted in 4 (8%) patients for a total of 46 (92%) treated with a minimally invasive approach (Table 3). There were 4 (8%) abdominal conversions to midline laparotomy, 1 due to intolerance of pneumoperitoneum, 1 due to presacral bleeding and 2 due to technical difficulty related to obesity. Complete mobilization of the splenic flexure was done in all cases and proximal inferior mesenteric pedicle ligation performed in 40 (80%) cases. Concomitantly with rectal resection, 2 resections of liver metastasis, 1 total colectomy and 2 protocolectomies were performed, all laparoscopically. Forty-seven anastomoses were fashioned, 40 (85%) mechanical and 7 (15%) handmade, predominantly side- to- end (72%), with a median distance to the dentate line of 20 mm (range 0–70 mm). All patients with a primary anastomosis had a protective loop ileostomy. The surgical specimen was extracted through a Pfannenstiel incision in 39 (85%) cases and pelvic drainage was placed in 24 (48%) patients. Median intraoperative blood loss was 100 mL (range 50–2000 mL), with only 1 patient requiring transfusion due to pre-sacral bleeding (Table 3). Median operative time was 285 min (range 202–445 min). Regarding the evolution of the learning curve, there was no difference in operative time in the first (median: 295 min; range 212–430 min) and last 25 patients (median 285 min; range 202–445 min).

Table 2 Preoperative staging and neoadjuvant therapy

Preoperative staging and neoadjuvant therapy		Patients ($n=50$)
Disease	Malignant	49 (98)
	Benign	1 (2)
Location, rectum, *n* (%)	1/3 superior	3 (6)
	1/3 medium	30 (60)
	1/3 inferior	17 (34)
Tumour extension (mm), median (range)		58 (5–120)
Distance to anal verge (mm), median (range)		20–120 (70)
cT	> T3	25 (50)
cN	Positive	25 (50)
CRM, *n* (%)	Free	39 (78)
	Threatened	3 (6)
	Invaded	7 (14)
	NA	1 (2)
EMVI, *n* (%)	Negative	44 (88)
	Present	5 (10)
	NA	1 (2)
CEA (ng/mL), median (range)		1.35 (0.5–296)
CRT, *n* (%)	No	26 (52)
	LCCRT	23 (46)
	SCRT	1 (2)
mTRG, *n* (%)	mTRG 1 e 2	11 (46)
	mTRG 3	3 (12)
	ND	10 (42)
	NA	26

cT and cN American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017, *CEA* carcinoembrinonary antigen, *CRM* magnetic resonance accessed circumferential resection margin, *EMVI* magnetic resonance accessed extramural vascular invasion, *CRT* chemoradiotherapy, *LCCRT* long course chemoradiotherapy, *SCRT* short course chemoradiotherapy, *mTRG* magnetic resonance accessed post-CRT Tumour Regression Grade [6], *NA* not applicable, *ND* not discriminated

Table 3 Surgical technique

Surgical technique		Patients ($n=50$)
CRT- surgery, weeks, median (range)		12 (7–22)
Abdominal approach, *n* (%)	Laparoscopy	42 (84)
	Laparotomy	4 (8)
	Robotic	4 (8)
Conversion, *n* (%)	Abdominal	4 (8)
	Transanal	0 (0)
Anastomosis, *n* (%)	Mechanical	40 (85)
	Hand-sewn	7 (15)
Anastomosis, *n* (%)	Side-to-end	34 (72)
	End-to-end	11 (24)
	Ileoanal pouch-anal	2 (4)
Anastomosis distance from dentate line, mm, median (range)		20 (0–70)
Specimen extraction site, *n* (%)	Pfannenstiel	39 (85)
	LIF	5 (11)
	Transanal	2 (4)
	NA	4
Operative morbidity, *n* (%)		
Abdominal approach	Pre sacral bleeding	1 (2)
Transanal approach	Vaginal lesion	1 (2)
	Urethral lesion	1 (2)
Stoma, *n* (%)	Loop ileostomy	47 (94)
	End colostomy	2 (4)
	End ileostomy	1 (2)
Drains, *n (%)*		24 (48)
Blood loss, mL, median (range)		100 (50–2000)
Operative time, minutes, median (range)		285 (202–445)

CRT chemoradiotherapy, *LIF* left iliac fossa, *NA* not applicable

Transanal approach

For the transanal approach, Lone Star® Retractor (Cooper Surgical, USA) and GelPOINT®Path Transanal Access Platform (Applied Medical, USA) were used. No conversion occurred. There were 2 (4%) intraoperative complications, 1 urethral and 1 vaginal lesion, both immediately repaired (Table 3).

Postoperative period and follow-up

There was a median length of stay of 7 days (range 3–42 days) with a readmission rate of 12% (6 patients). There was no postoperative mortality and 11 (22%) patients had Clavien-Dindo's IIIB morbidity [12]. There was no difference in the overall complication rate between the initial and late phase of the learning curve, with 5 versus 6 patients having Clavien-Dindo's IIIB morbidity, respectively.

In this study, the anastomotic leak was defined according to the International Study Group of Rectal Cancer, including radiological and clinical leak, pelvic and perianastomotic abscess [13]. There were 8 (17%) anastomotic leaks. Of these, 5 had to be treated with reoperation, 3 with transanal drainage, 1 with transabdominal drainage and only 1 with an end colostomy. 50% of patients that had anastomotic leaks had undergone neoadjuvant CRT. 46 (98%) patients maintained their anastomosis. Until the final date of this study, 44 (94%) had their ileostomies closed with a median time to closure of 31 weeks (range 2–67 weeks) (Table 4).

Pathological outcomes

Pathology reported 100% of R0 resection, free distal and circumferential margins, with a median node sampling of 19 nodes (range 4–52 nodes) and 49 (98%) good quality specimens graded as in mesorectal or intramesorectal plane [14] (Table 5).

Oncological outcomes

No patients were lost to follow-up and the median follow up time was 36 months (range 14–53 months).

Table 4 Postoperative period and follow-up

Postoperative period	
Hospital stay, days, median (range)	7 (3–42)
30-day mortality, *n* (%)	0 (0)
Readmission, *n* (%)	6 (12)
Postoperative complications (treatment), *n* (%)	22 (44)
Clavien-Dindo I	
Ileus	1 (2)
Clavien-Dindo II	9 (18)
Respiratory infection (AB)	2
Bacteriemia (AB)	1
Urinary tract infection (AB)	3
High output ileostomy (loperamide, omeprazol, codein)	1
Anastomotic leak, recto-vaginal fistulae (AB)	1
Anastomotic leak, pelvic abscess (AB)	1
Clavien-Dindo IIIA	
Anastomotic leak, presacral abscess (AB, endosponge)	1 (2)
Clavien-Dindo IIIB	11 (22)
Abdominal wall dehiscence (closure)	1
Pancreatic fistulae (drainage)	1
Intrabdominal haematoma (drainage)	2
Parastomal hernia (suture)	1
Jejunal fistulae (segmental resection)	1
Anastomotic leak	
Transanal drainage	3
Transabdominal drainage	1
End colostomy	1
Follow-up	
Ileostomy closure, *n* (%)	44 (94)
Time to stoma closure, weeks, median (range)	29 (2–67)
Adjuvant CT, *n* (%)	23 (46)

Complications classified according to Clavien-Dindo's classification [1]

AB antibiotic treatment, *CT* chemotherapy

Table 5 Pathological outcomes

Pathological outcomes	
Stage, *n* (%)	
Benign	1 (2)
T0N0M0	4 (8)
I	23 (46)
II	5 (10)
III	16 (32)
IV	1 (2)
Radicality, *n* (%)	
R0	50 (100)
Specimen quality, *n* (%)	
Mesorectal plane	40 (80)
Intramesorectal plane	9 (18)
Muscularis propria plane	1 (2)
Nodes, median (range)	19 (4–52)
Free margin, *n* (%)	
Distal	50 (100)
Circumferential	50 (100)
Tumour diameter, mm, median (range)	28 (15–45)

Pathological Staging according to the American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017; Specimen quality according to P. Quirke [3]

Table 6 Oncological outcomes

Oncological outcomes	
Follow-up time, months, median (range)	36 (14–53)
Time to local recurrence months, median(range)	15 (8–22)
Time to distant recurrence, months, median(range)	8 (1–17)
Local recurrence, *n* (%)	2 (4)
Anastomotic	1 (2)
Presacral	1 (2)
Distant recurrence, *n* (%)	10 (20)
Lung	4 (40)
Liver	2 (20)
CNS + bone	2 (20)
Periaortic nodes	2 (20)
Overall mortality, *n* (%)	4 (8)

CNS central nervous system

There were 2 (4%) cases of LR, one at 8 months, synchronous with distant metastasis, and another at 22 months in a patient with previous distant recurrence. Recurrences were presacral and anastomotic, respectively, with no pelvic lateral sidewall or multifocal pattern. The patient with a presacral recurrence had a suboptimal specimen with an incomplete mesorectum in the TaTME specimen. Median time to LR was 15 months (range 8–22 months) and patients had no metastasis at initial diagnosis.

There were 10 (20%) cases of metachronous distant disease after a median of 8 months (range 1–17 months). Patients who developed distant metastasis were initially in stage IV in 2 cases and stage III in 7. Patterns of distant recurrence related to metastasis in the lung, liver, central nervous system, bone and periaortic nodes (Table 6).

Overall, there were 4 deaths, all related to disease progression. One- and 3-year OS were 100% and 90%, respectively (Fig. 2a). 1- and 3-year DFS were 84% and 79%, respectively (Fig. 2b). 1- and 3-year distant recurrence-free survival were 84 and 79%, respectively (Fig. 2c).

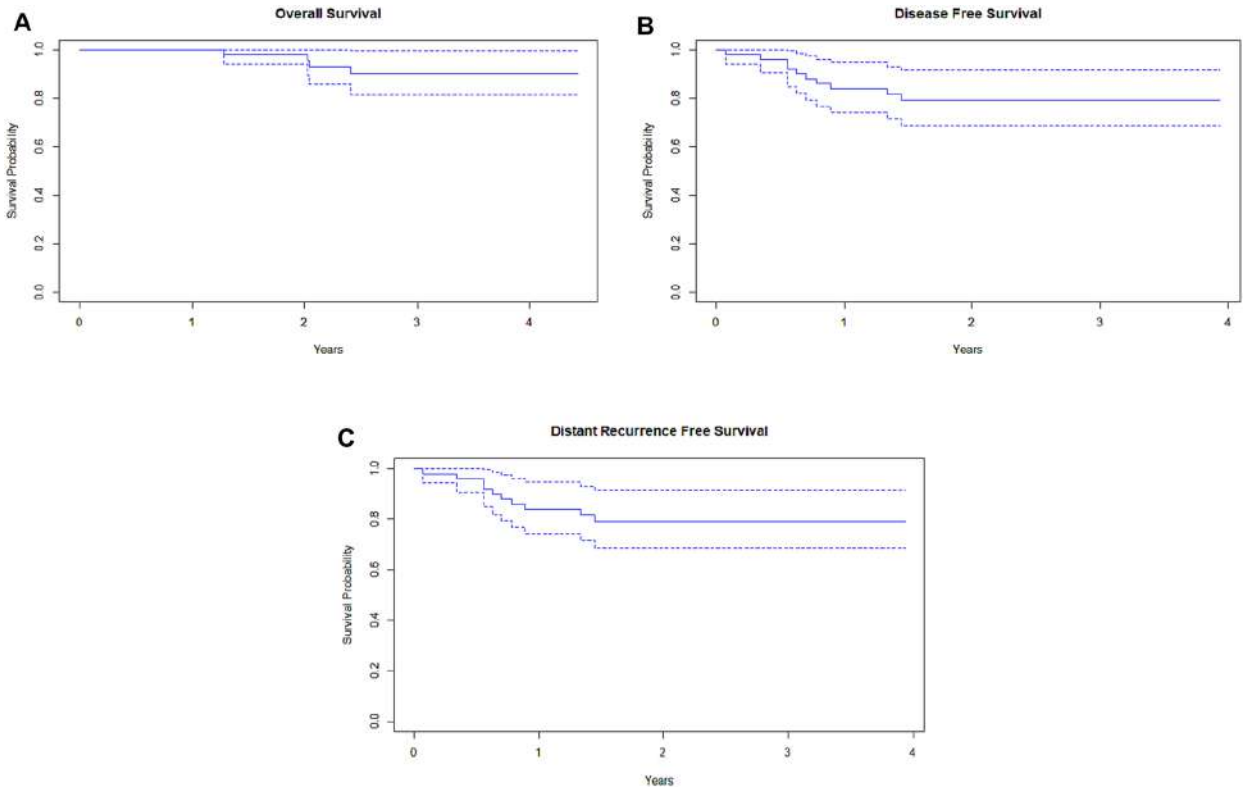

Fig. 2 Oncological Outcomes. Kaplan–Meier curves for **a** overall survival, **b** disease-free survival and **c** distant recurrence-free survival. One- and 3-year overall survival were 100% and 90% (**a**). 1 and 3-year disease-free survival were 84% and 79% (**b**). 1 and 3-year distant recurrence-free survival of 84 and 79% (1C)

Discussion

The application of laparoscopy to the treatment of rectal cancer has been a major technical leap that brought advantages in short- and long-term outcomes. Laparoscopic TME (lapTME) can, however, be very challenging in obese male patients with distal tumours due to the difficulties of pelvic dissection related to the limited operative field, decreased mobilization and stapling. In previous randomised controlled trials lapTME has been associated with high rates of conversion and anastomotic leak, incomplete mesorectum, invasion of circumferential resection margin and of distal margins [15, 16] with acknowledged impact on oncological outcomes [1, 14]

TaTME is the most recent surgical method developed to overcome technical difficulties in a pelvic approach. This reverse proctectomy has specific challenges associated with the change in anatomic perspective and the demands of a single-port technique. Likewise, it also brings new concerns, mainly reverse coning, vascular and urethral lesions.

When introducing new techniques in surgery there must be a scrutiny of outcomes for a safe implementation. It becomes imperative that surgeons report results and contribute to national and international validated databases. The aim of the present study was to present the mid-term clinical and oncological outcomes of the first 50 TaTME cases of our colorectal team, reflecting the learning curve.

In this study there were no cases of transanal conversion but there was, however, an intraoperative urethral lesion. This iatrogenic lesion, like pelvic sidewall vascular injury and CO_2 embolism, is associated with the technique [8, 9, 17].

Several authors report TaTME short-term results similar to or better than standard laparoscopic resection regarding conversion, anastomotic leak, involvement of distal and circumferential margins, mesorectal integrity, lymph node yield, operative time, blood loss, morbidity, length of hospital stay, readmission rates and function [18–22]. In our work, the anastomotic leak was defined according to the Rectal Cancer Study Group including clinical, radiological leak and perianastomotic/ pelvic abscess [13]. We present an overall early and late anastomotic leak rate of 17% that, despite being high, is concordant with what has been previously published [23]. In this 8 patients group, only one had their anastomosis taken down with definitive colostomy and all the rest had their loop protective ileostomies reversed.

Overall, intraoperative complications occurred in the initial stage of the learning curve (first 25 patients) with no differences in the evolution of the learning curve related to operative time and postoperative complications. Regarding pathological outcomes, specimen quality was good with 49 (98%) graded as in mesorectal or intra mesorectal plane and 100% with R0, clear distal and circumferential margins.

Although it seems to be well-established that short-term clinical outcomes are good there are still inconsistencies regarding oncological ones. Several authors have reported good oncological outcomes but mostly with a short follow-up time [24–30]. Likewise, studies that compare survival between lapTME and TaTME also present good oncological outcomes but, again, with only short-term follow-up [31–36].

In fact, few studies report on more than 2-year oncological outcomes. A recent multicenter study on 211 TaTME patients demonstrated 3-year OS of 93%, DFS of 80% and 6% of LR [19]. Perdawood et al. [37] published on 200 TaTME patients and, with a follow-up of 2 years, found 90% OS, 81% DFS, 5% of LR and 12% of distant metastasis. Marks et al. studied 373 patients that underwent Trans Abdominal Trans Anal approach (TATA) with the abdominal dissection performed through laparoscopic, pure transanal, open or robotic approach. With a mean follow-up of 66 months (range 0–300 months), 5-year OS was 90% and LR was 7.4% [38, 39]. Recently, Hol et al. [40] reported that 159 TaTME patients at 3 and 5 years had 84% and 77% OS, 92% and 81% DFS and 2% and 4% LR, respectively. Finally, in a controlled trial with 100 patients randomized to lapTME and TaTME, Denost et al. [41] reported no significant difference between groups regarding 5-year LR or DFS.

Until now, the fact that most studies only express short-term oncological outcomes has not allowed definitive conclusions do be drawn. In addition, recent literature has raised concerns about the oncological safety of TaTME with publications reporting early multifocal pelvic cavity and sidewall recurrence [42, 43].

In this setting, the present study had the objective of investigating 3-year oncological outcomes of our first 50 TaTME patients, reflecting the learning curve. With a median follow-up of 36 months (range 14–48 months), we report 2 (4%) cases of LR, occurring at 8 and 22 months, none multifocal or related to the pelvic sidewall. The first of these cases related to a patient with an intraoperative urethral lesion and a suboptimal specimen who developed a presacral recurrence at 8 months, which shows the importance of specimen quality and surgical technique.

In our cohort, distant metastases were found in 10 (20%) patients after a median of 8 months (range 1–17 months), 2 of whom had stage IV disease at initial diagnosis and the other 7 stage III. Three-year OS and DFS were 90% and 79%, respectively. Although limited by the small number of patients, we intended to show the outcomes of the learning curve of TaTME, accepted to be at least 20 cases per surgeon [10, 44, 45]. Our results concerning the oncological safety of TaTME parallel the published outcomes for lapTME published so far.

TaTME cannot be seen as a technique to replace either laparoscopic or open approaches but rather as another option available in the surgical armamentarium, indicated in particular cases, mainly obese male patients with distal tumours. We still cannot fully comprehend the disparity of results between publications regarding oncological outcomes. In this context, it becomes imperative to contribute to a better understanding of this new technique by reporting one's results.

Conclusions

TaTME can produce safe mid-term oncological results, comparable to those reported for open and laparoscopic TME. Our study also shows how demanding this new technique can be and consequently the need for audited data and standardized implementation.

Author contributions SO: study conception and design, acquisition, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article. BC: analysis and interpretation of the data, critical revision of the article for important intellectual content final approval of the article. MPF: analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article. DA: analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article. PR: acquisition, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article. DF: analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article. RM: critical revision of the article for important intellectual content, final approval of the article.

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Availability of data and material The datasets analysed during the current study are available in the Hospital Beatriz Ângelo e Hospital da Luz informatics hospital database (Soarien), obtainable from the corresponding author on reasonable request.

Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest or disclosures.

Ethical approval The present study was approved by the Ethics Committee and Institutional Review Board of Hospital Beatriz Ângelo and Hospital da Luz with no formal Informed consent required due to its methodology and anonymity. The study protocol was performed in

accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

Informed consent All patients accepted this technique through informed consent.

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Techniques in Coloproctology

Transanal versus Laparoscopic total mesorectal excision. Comparative study of long-tem oncological outcomes

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Abstract:	Purpose:Transanal total mesorectal excision is the most recent approach developed to improve pelvic dissection in surgery for mid and low rectal tumors.There are still inconsistencies regarding the technique's oncological results. This study analyses clinical and oncological outcomes of the learning curve of TaTME (TaTME) in comparison to a matched group of patients treated by laparoscopic TME (lapTME). Methods:Rectal cancer patients submitted to TaTME and lapTME in two Portuguese colorectal unitsbetween March 2016 and December 2018 were eligible. Primary endpoints were 4-year overall survival, disease-free survival and local recurrence. Secondary endpoints were clinical and pathological outcomes. Results:47 patients underwent TaTME and 44 lapTME. No differences were observed concerning baseline characteristics, emphasizing their comparability. In the TaTME group there were more loop ileostomies performed (33 LapTME versus44 TaTME, p=0.018) and more hand-sewn anastomosis (0 LapTME versus7 TaTME, p=0.016) p=0.016), with a trend for lesser distance to the anal verge (35mm lapTME versus20 TaTME, p=0.061). There were no differences in mortality, overall complications, Clavien-Dindo≥ IIIB morbidity, readmissions and stoma closure. Also, groups were similar in relation to pathological stage, specimen quality, margins, ressecability and node sampling. Finally, no disparities were noted in oncological outcomes, namely local and distant recurrence, 4-year overall survival and disease-free survival. Conclusions:Even reflecting the learning curve of a new technique, TaTMEcan be comparable to lapTME, with similar long-term oncological outcomes. It has, however, a demanding learning curve and significant risk for morbidity,for witch should be selectively considered.

INTRODUCTION

The impact of the surgical options in rectal cancer (RC) patient's outcomes is undeniable and impressive improvements have been introduced in the last decades. In fact, we evolved from one single technique performed in all RC patients to a multitude of procedures, individually selected according to patient performance status, oncological risk or even response to neoadjuvant therapies. Still, the gold standard treatment of this malignancy is total mesorectal excision (TME) that can, however, be very challenging, especially in a particularly demanding group of obese male patients with narrow pelvis and distal tumors. In fact, these characteristics are the principal risk factors for positive circumferential resection margin (CRM) and intraoperative technical difficulties [1],[2],[3].

Transanal total mesorectal excision (TaTME) was developed to overcome difficulties in dissection of the pelvic compartment allowing a better visualization of the recto-prostatic/ recto-vaginal septum and neurovascular bundles, with improved margin definition and avoiding stapling distal to the tumour [4],[5]. Good TaTME short-term clinical outcomes have already been accepted but there are still inconsistencies regarding oncological ones. This study had the primary goal to investigate the long-term oncological outcomes of the introduction of TaTME in a Portuguese colorectal Group and to compare it to a matched group of patients treated by laparoscopic TME (lapTME).

MATERIALS AND METHODS

This was a retrospective observational study. It compared consecutive patients with mid and low RC stage I-IV, American Joint Committee on Cancer (AJCC) submitted to TaTME between March 2016 and December 2018 in Hospital Beatriz Angelo and Hospital da Luz in Lisbon to a matched group of patients treated with lapTME in the same institutions. These first TaTME patients reflected the learning curve of the technique [6].

The unit's volume of rectal radical resection during the elected study period is shown (Fig 1). Prior to TaTME implementation, surgeons underwent observation of live procedures, hands-on modular training courses and proctored learning. Data regarding TaTME cases was introduced in the International TaTME Registry.

Tumors were defined as in the mid or low rectum if located between 5-10 cm and less than 5 cm from the anal verge, respectively, by magnetic resonance (MR) and rigid sigmoidoscopy.

Pathological specimen plane was defined according to Quirke *et al* as '*muscularis propria* plane', mesorectal plane' or the 'intramesorectal' [7].

In this study, anastomotic leak was defined according to the International Study Group of Rectal Cancer, including radiological and clinical leak, pelvic and perianastomotic abscess [8].

Post-operative morbidity was assessed according to Clavien-Dindo Classification [9] and included all complications related to the initial surgery, even after 30 days. Data was obtained from the hospital's electronic database.

Primary endpoints were oncological outcomes, namely overall survival (OS) disease-free survival (DFS) and local recurrence (LR). Secondary endpoints were clinical, pathological outcomes and parameters of specimen quality.

Statistical analysis

In this retrospective study, continuous variables were reported as n, median, first and third quartiles (Q1, Q3). To compare characteristics between patients that performed lapTME or TaTME, independent t test for equal and unequal variances, proportion test, chi-squared test and Fisher exact test were applied, as appropriate. Analysis time to event data was performed through Kaplan–Meier (KM) curves. Overall survival (OS) was calculated considering surgery date until death date. Disease-free survival (DFS) was estimated considering surgery date until the appearance of recurrence, local or distant. Local recurrence-free survival (LRFS) was assessed measuring time from surgery date till the appearance of LR. Finally, distant progression -free survival (DPFS) was calculated considering surgery date until the appearance of distant progression. Estimated median time to event, 25^{th}- 75^{th} percentiles and correspondent 95% confidence interval (CI) were presented. Probability of survival for these time points and respective 95% CI were also disposed. For comparing survival times between groups log-rank test was used. Significance level was set at $p \leq 0.05$. Data was analyzed with R (version 4.0.2, 2020-06-22, "Taking Off Again").

RESULTS

Patient clinical parameters

During the elected period, a total of 47 patients were submitted to TaTME and 44 to lapTME with predominance of male gender, performance status (PS) Eastern Cooperative Oncology Group (ECOG) 0 and ASA (American Society of Anesthesiologists) 2 in both groups. There were no significant differences between cohorts in terms of baseline characteristics (Table 1).

Pre-operative staging and neoadjuvant therapy

The majority of patients were treated for cancer, 46 in the TaTME group and 41 in the lapTME. One patient had TaTME for an endoscopically non-resectable tubulovillous adenoma with high-grade dysplasia and 3 patients underwent lapTME for ulcerative colitis with high-grade dysplasia.

Patients with RC were staged with pelvic MR and thoraco-abdomino-pelvic computed tomography (CT) except for 2 that underwent endorectal ultrasound (ERUS) due to the presence of metallic prosthesis. There were no differences between groups regarding tumour location, extension, distance to the anal verge, cT, cN, cM, clinical stage, CRM, extramural vascular invasion (EMVI) and carcinoembryonic antigen (CEA). The majority were patients in stage III, without EMVI and with free CRM. Likewise, the mainstream of patients in both groups underwent neoadjuvant therapy (32 lapTME versus 23 TaTME, p=0.686), mostly with a long course chemoradiotherapy (LCCRT). There were no differences between TaTME and lapTME groups regarding tumour characteristics, stage, neoadjuvant regimen chosen and tumour regression grade assessed by MR (mrTRG) (Table 2).

Surgical technique

All patients had preoperative mechanical oral bowel preparation and underwent surgical procedure in a median of 12 weeks after chemoradiotherapy (CRT) (range 10-13 and 11-13, p=0.266 in TaTME and lapTME groups, respectively).

The TaTME procedure was performed with 2 teams, transabdominal and transanal, working synchronously, with complete mobilization of the splenic flexure in all cases, using *Lone Star® Retractor (Cooper Surgical, USA*) and *GelPOINT®Path Transanal Access Platform (Applied Medical, USA)* for the transanal approach. This procedure was done through laparoscopy in 39 (82%) patients and robotically in 4 (9%) cases, in a total of 43

(91%) through minimally invasive approach. With no transanal conversions, there were 4 (9%) abdominal conversions to midline laparotomy, 1 due to pre-sacral bleeding, 1 for pneumoperitoneum intolerance, and 2 for obesity-related technical difficulties. Concurrently with the protectomy, 4 protocolectomies and 2 liver metastasis resections were made, also by laparoscopy.

There were no differences between groups related to the number of anastomosis performed with a predominance of mechanical, side-to-end anastomosis in both. Groups were comparable regarding specimen extraction site, intraoperative blood lost, complications and operative time. There were, however, more hand-sewn anastomosis in TaTME group (0 lapTME *versus* 7 TaTME, p=0.016) with a trend for a lesser distance from the anal verge (35 mm lapTME *versus* 20 TaTME, p=0.061). Also, more loop-ileostomies (33 LapTME versus 44 TaTME, p=0.018) were used in the TaTME group. On the contrary, more pelvic drains were placed in the lapTME cohort (30 lapTME *versus* 22 TaTME, p= 0.039) (Table 3).

Post-operative period and Follow-up

There were no differences between groups related to 30-day mortality, overall complications rate, morbidity higher than Clavien-Dindo IIIB or anastomotic leakage (11% lapTME *versus* 17% TaTME, p= 0.367). Of these leaks, in the lapTME group, 4 patients underwent surgical re-exploration with 2 end colostomies, one transabdominal and one transanal drainage. In the TaTME group, 6 patients had to be re-operated with one end colostomy, one trans-abdominal and 4 transanal drainages. Overall, 36 (95%) and 43 (98%) patients maintained their anastomosis in the lapTME and TaTME groups, respectively.

No differences were found regarding length of hospital stay, readmission rate, stoma closure and number of patients undergoing adjuvant therapy. Until the final date of this study, 29 (88%) and 37 (84%) had their ileostomies closed in the lapTME and TaTME groups, respectively (Table 4).

Pathological Outcomes

There were no differences between groups related to pathological stage, circumferential, proximal and distal margins, ressectability, node sampling and specimen quality. Both techniques showed good quality specimens with appropriate margins and lymphadenectomies (Table 5).

Oncological outcomes

Median follow up time was 33 (17-56) and 36 (28-48) months in the lapTME and TaTME groups, respectively (p=0.464). Because the majority of patients in the TaTME group only achieved 4 years of follow-up, we report 4-year oncological outcomes.

In the lapTME group there was one (2%) case of LR at 16 months, in the presacral area in a patient with previous distant disease. There were 4 (10%) cases of distant progression (DP) after a median of 15 (6-23) months in patients that were initially stage III (3 cases) and IV (1 case). In this group there were 7 (16%) deaths, 1 due to disease progression, 2 to complications of index surgery and 4 to non-oncological co-morbidities (vascular, liver and cardiac insufficiency) (Table 6). Four-year OS and DFS were 82% (CI 0.713-0.953) and 91% (CI 0.825-1), respectively. Also, 4-year DPFS and LRFS were 91% (CI 0.825-1) and 96% (CI 0.882-1), correspondingly (Fig. 2).

In the TaTME group there were 2 (4%) cases of LR at 8 and 22 months. Recurrences were presacral and anastomotic, respectively, with no pelvic sidewall pattern. The patient with a presacral recurrence had synchronous hepatic metastasis and a suboptimal specimen with an incomplete mesorectum following a procedure with long operative time and an intraoperative urethral lesion. In this group there were 10 (21%) cases of distant disease, one synchronous with LR and 9 metachronous, after a median of 8 (7-11) months. Patients who developed distant metastasis were initially in stage IV in 2 cases and stage III in 7. Metastatic disease involved the lung, liver, central nervous system, bone and periaortic nodes. In the TaTME group there 5 deaths, all related to distant disease progression (Table 6). Four-year OS and DFS were 86% (CI 0.760-0.985) and 78% (CI 0.666-0.910), respectively. Finally, 4-year DPFS and LRFS were 78% (CI 0.666-0.91) and 94% (CI 0.860-1), correspondingly (Fig. 2).

Overall, there were no differences between lapTME and TaTME groups related to mortality (p=0.543), LR (p=0.999) and DP (p=0.158). Likewise, cohorts presented similar 4-year OS, DFS, LRFS and DPFS (p=0.4, p=0.1, p=0.7 and p= 0.1 respectively) (Fig. 2).

DISCUSSION

Despite the great advance in rectal surgery brought by lapTME in terms of short and long-term outcomes, this technique can be very demanding, particularly in a specific group of patients with obesity, distal bulky tumors. In previous randomized controlled trials lapTME for mid and low RC has been associated with high anastomotic leak, conversion to laparotomy and suboptimal TME specimens, with known deleterious oncological consequences [1],[6], [9],[10]. The difficulty relates to operating in the low pelvic compartment with restricted working space, limited vision and maneuverability.

Surgeons have tried to developed alternatives to overcome these problems and TaTME was introduced in 2010 to improve pelvic approach [4]. The technique has several potential advantages, namely a better view of the prostate and recto-vaginal septum with ability to decide whether to stay in front or behind Denonvilllier's fascia in anterior tumors, better visualization of neurovascular bundles and pelvic floor muscles, reduced specimen manipulation due to the pneumorectum aid in dissection and surgeon's determination of the appropriate distal margin [5],[12],[13]. Potential gains from this technique are an easier dissection in the male narrow pelvis, a decrease in conversion, an increase in sphincter saving resections, better anastomotic techniques with subsequent lower morbidity, improved specimen quality and a decrease in surgical site infection [12],[13]. Also, TaTME dos not require stapling of the rectum distally to the tumour, avoiding imperfect firing (due to the limitation of staplers 45º angulation), "dog ears" and crossing of staple lines. In classic laparoscopy, low pelvic tumors frequently need several staplings, with known association with anastomotic leak [16]. However, TaTME has specific challenges associated with the change in anatomic perspective and the demands of a single-port technique. Likewise, it also introduced new complications, not associated with the open or laparoscopic approaches, namely urethral injuries, carbon dioxide embolism and reverse coning [15],[16].

TaTME was started in our Colorectal Unit in March 2016. Prior to the introduction of the technique, institutional protocols and procedural guidelines were developed and surgeons underwent hands-on courses, observation of live procedures, didactic learning through *iLapp* platform, with the first cases performed mentored by international proctors.

Having already studied the short- term outcomes [19], the present study had the objective of analyzing the long-term clinical and oncological outcomes of the learning curve of TaTME in our institution. It also intended to compare these outcomes to the ones of a matched group of patients treated with lapTME by the same surgeons.

In this study TaTME and lapTME groups were comparable in terms of demographic and clinical characteristics, with no differences in terms of gender, age, BMI, PS, ASA scores, baseline tumour characteristics, neoadjuvant therapy and subsequent response. Groups were also surgically comparable with the exception that TaTME patients had more hand-sewn anastomosis (0 lapTME *versus* 7 TaTME, p= 0.016) and loop ileostomies performed (33 lapTME *versus* 44 TaTME, p= 0.018). LapTME had more drains placed in the pelvis (30 lapTME *versus* 22 TaTME, p= 0.039).

So far, published literature show that TaTME has short-term clinical outcomes similar or better than lapTME regarding conversion, anastomotic leak, distal and circumferential margins, mesorectal integrity, lymph node yield, operative time, blood lost, morbidity, length of hospital stay (LOS) and readmission rates [20],[21],[22]-[29],[30],[31]. Outcomes regarding function are still controversial, although most studies present comparable results [32]-[34].

In our work, we also obtained similar early outcomes, namely LOS, re-admission rates, overall complications, Clavien-Dindo higher than IIIb morbidity and overall leak rate. Although we report that 8 patients in the lapTME group had to be re-operated, overall

anastomotic leak was 11%. Likewise, in the TaTME cohort, while 11 patients had a re-intervention, only 6 were due to anastomotic leak. Although not statistically different between cohorts (11% lapTME *versus* 17% TaTME, p= 0.367), the anastomotic leak rate in TaTME group, probably a consequence of the initial learning curve, is worrisome and must be mitigated. Also, the lower ileostomy rate in lapTME might explain the fatal outcome of 2 anastomotic leaks. Regarding pathological outcomes, there were no disparities between groups in stage, ressectability, node sampling, circumferential, proximal and distal margins and specimen quality.

Although TaTME short-term clinical outcomes seem to be well established, inconsistencies remain regarding oncological outcomes and some authors have even reported disturbing results of early sidewall and multifocal pelvic cavity recurrence [35]. In this work, we did not experience these negative outcomes, in our opinion possibly a consequence of several reasons, namely the use of a non-standardized procedure, surgeons endorsing TaTME prior to a proficient learning curve or even technical differences between surgical teams. In fact, we still cannot fully comprehend the discrepancy of results between publications.

So far, very few studies that report on TaTME have a follow-up longer than 3 years. Marks *et al* analyzed 373 patients submitted to Trans Abdominal Trans Anal approach (TATA) with the abdominal dissection done through pure transanal, laparoscopic, robotic or open approach. With 66 (range 0–300) months of mean follow-up, 5-year LR was 7.4% and OS was 90% [36]. Recently, Hol *et al* reported on 159 TaTME patients with 5 year 4% LR, 77% OS and 81% DFS [37]. Lastly, in a trial with 100 patients randomly assigned to TaTME and lapTME, Denost *et al* described no difference in 5-year LR or DFS between groups [38]. The fact that most other studies only report short-term oncological outcomes has not allowed definitive conclusions.

In our study, the LapTME group had one (2%) case of LR, happening at 16 months, in the presacral area in a patient with prior distant progression. In the TaTME group there were 2 (4%) cases of LR, pre sacral and anastomotic, none multifocal or in the pelvic sidewall. Overall, no differences were perceived regarding LR (p=0.999). In the lapTME group, 4-year OS and DFS were 82% (CI 0.713-0.953) and 91% (CI 0.825-1) similar to the 86% (CI 0.760-0.985) and 78% (CI 0.666-0.910) DFS presented by the TaTME group. Also, lapTME 4-year LRFS and DPFS were 96% (CI 0.882-1) and 91% (CI 0.825-1) parallel to the 94% (CI 0.860-1) and 78% (CI 0.666-0.910) of the TaTME group. Overall, no differences occurred related to 4-year OS, DFS, LRFS and DPFS (p=0.4, p=0.1, p=0.7 and p= 0.1 respectively).

The main limitation of this work is its non-randomized methodology and also the non-inclusion of data regarding functional outcomes. Notwithstanding, the similarity observed between groups in respect to baseline characteristics emphasizes the comparability of the groups. Also, our follow-up is longer than what most studies published so far.

The question is no longer “can good results be obtained by gifted surgeons appropriately trained?” It has moved on to “can this technique be performed reliably, safely and with good outcomes by the average surgeon on the common patient? Reflecting the learning curve of the technique, accepted to be 20-25 cases per surgeon [39], our results show similar pathological and oncological outcomes between lapTME and TaTME, in accordance to what has been the generalized perception of the technique. It must be emphasized, however, that TaTME has a demanding learning curve and significant risk for morbidity. For its safe introduction it is fundamental to understand the different anatomical perspective it involves [40],[38],[39], to implement an intensive multimodal training with hands-on cadaver training, proctored application, following international guidelines [36],[37],[40]-[42] and apply it to carefully selected patients. Also, it is imperative that surgeons are experienced not just in laparoscopy but also in single-port and low pelvic surgery. Finally, the transparent scrutiny of the TaTME technique relies in reporting one’s results, participating in ongoing multicenter randomized trials and in international Registries.

The fact is that, whatever technique is used to performed low RC surgery, it requires advanced skills and optimal results can only be achieved with adequate training and continuous evaluation of outcomes to ensure they improve as experience grows.

CONCLUSION

Intended to analyze the outcomes of the introduction of TaTME in a colorectal Group, our study showed that the technique can produce long-term oncological safe outcomes, comparable to lapTME. Results also reflect how demanding this new technique can be and the consequent need for a strict patient selection and proper learning curve. In our opinion, TaTME does not intent to replace other established approaches to rectal surgery but to add a new alternative to address difficult cases.

Conflicts of interest/ Competing interests: the authors declare no conflicts of interest or disclosures.

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REVIEW

Oncological outcomes of local excision compared with radical surgery after neoadjuvant chemoradiotherapy for rectal cancer: a systematic review and meta-analysis

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Abstract
Background Low rectal cancer is conventionally managed with neoadjuvant chemoradiotherapy (CRT) followed by radical surgery (RS). In patients who refuse a stoma or are unfit for RS, an alternative approach may be the use of pre-op CRT and local excision (LE) where tumours are responsive. The aim of this systematic review is to determine whether differences exist in local recurrence (LR), overall survival (OS) and disease-free (DFS) survival between patients treated with CRT+LE and CRT+RS.
Methods A literature search was performed using MEDLINE/PubMed/Ovid databases and Google Scholar between 1946 and 2013. Studies comparing outcome following LE and RS post-CRT were included. A pooled analysis was carried out using the Mantel-Haenszel statistical (random effects) model to identify differences in LR, OS and DFS between CRT+LE and CRT+RS.
Results Eight studies were suitable for pooled analyses of LR whereas five and four studies were analysed for OS and DFS, respectively. When RS was used as the reference group, LR rate was higher in the LE group. However, this was non-significant (odds ratio (OR) 1.29, confidence interval (CI) 0.72–2.31, p=0.40). Similarly, no difference was observed in 10-year OS (OR 0.96, CI 0.38–2.43, p=0.93) or 5-year DFS (OR 1.04, CI 0.61–1.76, p=0.89). There was evidence of publication bias in studies used for DFS. Subgroup analysis of above outcomes in T3/any N stage cancers showed no difference in LE versus RS.
Conclusion In the current evidence synthesis, there was no statistical difference in the LR, OS and DFS rates observed between patients treated with LE and RS for rectal cancer post-CRT. LE post-CRT may represent a viable alternative to RS for some patients wishing to avoid RS. However, further randomised studies are required to confirm these results.

Keywords Low rectal cancer · Chemoradiotherapy · Local excision · TEMS · Anterior resection

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Introduction

Locally advanced rectal cancer is conventionally treated with neoadjuvant chemoradiotherapy (CRT), followed by radical surgery. In the UK, the National Institute of Clinical Excellence (NICE) recommends that low-risk rectal cancers such as T1–T2 and T3a with N0 rectal cancers can be managed by surgery alone. Moderate risk rectal cancers, including T3b tumours, may be considered for short-course radiotherapy. High-risk tumours including threatened circumferential resection margins or encroaching into the intersphincteric plane/levator muscle plate are recommended for long-course chemoradiotherapy. Radical surgery includes total mesorectal excision (TME) with anterior resection or abdominoperineal resection performed by either a laparoscopic or traditional open approach [1, 2]. Some investigators have advocated that patients demonstrating complete response to CRT may be safely observed without surgical resection [3]. TME with or without preoperative CRT is currently the standard of care for mid and lower third rectal cancers and has a local recurrence rate and 5-yeaar overall survival of 6–8 and 76 %, respectively [4, 5]. TME is however a major surgical procedure with

associated risk of mortality and significant associated morbidity including anastomotic leakage, injury to genitourinary nerves or ureters, mortality, as well as variable bowel functional outcome [5, 6]. Moreover, a proportion of patients undergoing rectal cancer surgery will require either a temporary or permanent stoma [7]. Acceptable oncological outcome has been reported in selected patients treated with only CRT with complete clinical response and observation. Habr-Gama and colleagues followed up 99 patients who had complete clinical response (cCR) for at least 12 months after CRT for rectal cancer and reported an overall recurrence rate of 13.1 % (including 5 % endorectal, 7.1 % systemic and 1 % combined recurrence) [8]. The same group in 2014 reported a 31 % local recurrence rate amongst cCR patients. Of 90 patients, 23 had local recurrence only, 5 had combined systemic and local recurrence and 8 patients had only systemic recurrence. However, overall recurrence was 40 % [9, 10]. Dedemadi and Wexner in a review reported excellent long-term survival in patients who had a complete clinical response after neoadjuvant CRT treated both with surgery and non-surgical management [9].

An alternative option to radical surgery in certain circumstances is local excision (LE) following CRT. LE can be performed using minimally invasive techniques such as transanal endoscopic microsurgery (TEMS) [11] and transanal minimal invasive surgery (TAMIS) [12]. Endoscopic assessment and MR imaging re-staging has been shown to be sensitive tools that facilitate the selection of patients suitable for LE [13]. If margins are positive after LE, immediate radical surgery (RS) may be considered in suitable patients [13]. Therefore, LE strategies may obviate the need for RS as a primary procedure.

The aim of this systematic review and meta-analysis is to determine whether there are differences in local recurrence, overall survival (OS) and disease-free survival (DFS) rates between patients undergoing LE and RS for rectal cancer post-CRT. A secondary aim was to analyse the differences in outcome depending on pre-treatment T stage.

Methods

A literature search was performed using MEDLINE/PubMed/Ovid databases and Google Scholar from 1946 to July 2013. The following keywords were used: rectal cancer combined with surgical resection, LE and total mesorectal, abdominoperioneal resection, RS, without any restriction on language. Searching was restricted to human studies. In instances where there was more than one publication by the same investigating group using the same study population, the latest study was used unless studies referred to different patient populations. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram was used to illustrate the search methodology.

Inclusion criteria

Studies were included if they investigated local recurrence, OS and DFS rectal adenocarcinoma with any T stage with any nodal status. Only studies that involved a direct comparison between LE and RS after chemoradiotherapy were included.

Exclusion criteria

Studies that did not describe the above outcomes, reviews, editorials or where there was insufficient information provided for data extraction were excluded. Similarly, studies involving patients undergoing surgery for recurrent disease or included patients that had metastatic disease from the outset were excluded.

Data extraction and quality assessment

Data were extracted by two investigators, IS and AA, using a predefined proforma. We collected data on patients undergoing local and radical resection and investigated the primary outcomes of disease recurrence, OS and DFS. Disagreements were resolved by discussion with the third investigator. Where data extraction was not possible due to insufficient information, the study was excluded. The NICE for Quality Assessment of Case Series was used to evaluate the quality of studies (NICE, 2014, www.nice.org.uk).

Statistical method and publication bias

All data were analysed using Review Manager 5 (RevMan, versions 5.2.1, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). The Mantel-Haenszel method, using random effects analysis, was used to evaluate risk (odds ratio) of cancer recurrence, OS and DFS between the two groups (LE and RS). A funnel plot applying Egger's test was charted to evaluate the risk of publication bias amongst the included studies.

Results

The search retrieved a total of 84 articles (Fig. 1). Three further articles were identified through manual searching. Duplicates were removed and review articles were excluded, leaving a total of 66 abstracts for screening. Within these, 50 articles were excluded as the studies did not provide specific data on surgical or oncological outcome leaving a total of 16 articles. Of these, a further eight were excluded as they did not compare LE with RS, leaving a total of eight articles. Of these eight (Table 1), seven had pooled their recurrence and survival outcomes across different tumour stages. Further subgroup

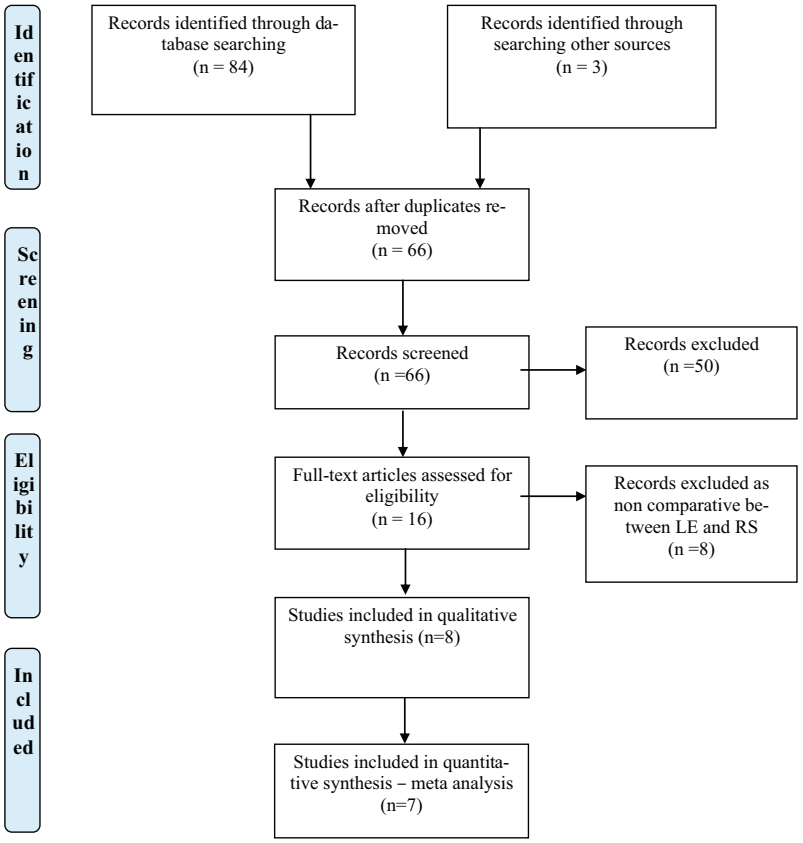

Fig. 1 A PRISMA diagram outlining search strategy and selection of included studies

analysis for local recurrence of T3 (any N stage) cancers resulted in only two studies, and no isolated outcomes of interest were reported for T1 or T2 stage. Only one study [11] was a randomised controlled trial (RCT) and was therefore reported separately and not included in meta-analysis.

Local recurrence

Of the seven included studies eligible for inclusion in the local recurrence analyses, three studies were from the USA [14–16] and one each from Italy [17], Brazil [18], South Korea [19] and Israel [20]. The total patient population in the pooled analysis was 1,301 with 157 patients in the LE group and 1,144 patients in the RS group. Across the seven studies, four [14, 16, 18, 19] observed a higher recurrence rate in the LE group, while three [15, 17, 20] observed a higher rate of recurrence in the RS group (Table 2). The RCT [11] reported local recurrence in four patients (4/50, 8 %) in the LE group, compared with three (3/50, 6 %) in the RS group. Pooling of data excluding the RCT (Fig. 2) from relevant studies demonstrated that a total of 16 patients (16/157, 10.1 %) had local cancer recurrence in the LE group and 95 (95/1,144, 8.0 %) in the RS group. The pooled odds ratio of local cancer recurrence was 1.29 (confidence interval (CI) 0.72–2.31, p=0.40). There was no heterogeneity in the pooled analysis (I^2=0 %). Subgroup analysis (Fig. 3) of the studies [15, 16] investigating T3 and any N stage cancers only revealed no significant difference (odds ratio (OR) 1.28, CI 0.56–2.91, p=0.56) in local recurrence rates between LE (7/73, 9.5 %) and RS (68/878, 7.7 %).

Overall survival

Four studies were selected for 10-year survival pooled analysis [15, 17, 19, 20]. The total population included in the analysis was 585 patients (LE, n=80; RS, n=505). The 10-year OS (Fig. 4) in the LE group was 83.5 % (67/80) and 79.0 % (399/505) in the RS group. All studies showed better survival in the LE group but failed to reach statistical significance. The RCT [11] showed no significant difference in OS in LE versus RS (p=0.609). Pooled analysis did not demonstrate a difference in OS between LE and RS (OR 0.96, CI 0.38–2.43, p=0.93). Further subgroup analysis for T3 (and greater) tumours was not possible as only one study reported the required outcome.

Disease-free survival

Five studies provided data on 5-year DFS for pooled analysis [15–17, 19, 20]. The total population in this subgroup was

Table 1 Study characteristics and patient demographics

Study (year)	Type of study	Study period	Country	LE group (*n*)	RS group (*n*)	Sex (*n*)		Mean age (range)		Median follow-up in months (range)	
						LE	RS	LE	RS	LE	RS
Bannon et al. [14] (1995)	Prospective cohort	1993–1995	USA	44	65	M, 22 (50 %) F, 22 (50 %)	M, 43 (66.2 %) F, 22 (33.8 %)	68 (42–86)	59 (29–83)	36 (2–94)	40 (1–107)
Bonnen et al. [15] (2004)[a]	Prospective data collection; retrospective analysis	1990–2002	USA	26	405	M, 17 (65 %) F, 9 (35 %)	M, 247 (61 %) F, 158 (39 %)	60 (35–80)	59 (21–88)	42 (4.5–109)	32 (3–113)
Callender et al. [16] (2010)	Retrospective cohort	190–2008	USA	47	473	–	–	62.5 (48.3–76.7)	58.3 (45.8–70.3)	63 (9–178)	59 (4–172)
Caricato et al. [17] (2006)[a,b]	Prospective cohort	1997–2002	Italy	8	22	M, 22 (73.4 %) F, 8 (26.6 %)	64 (49–75)	37 (24–66)			
Habr-Gama et al. [18] (1998)	Prospective cohort	1991–1996	Brazil	9	78	M, 68 (57.6 %) F, 50 (42.4 %)	57 (21–82)	36 (8–67)			
Huh et al. [19] (2008)[a,b]	Retrospective cohort	1994–2005	South Korea	9	64	M, 5 (55.6 %) F, 4 (44.4 %)	M, 41 (64.1 %) F, 23 (35.9 %)	55 (42–69)	54 (31–80)	91 (50–127)	55 (3–132)
Kundel et al. [20] (2010)[a,b]	Retrospective cohort	1997–2007	Israel	14	37	M, 160 (50 %) F, 160 (50 %)	70 (60–79)	68 (40–78)	67 (28–86)	48 (5–123)	
Lezoche et al. [11] (2012)[a,b]	Randomised control trial	1997–2004	UK	50	50	M, 30 (60 %) F, 20 (40 %)	M, 34 (68 %) F, 16 (32 %)	66 (58–70)	66 (60–69)	115 (102–133)	115 (89–143)

LE local excision, *RS* radical surgery, *M* male, *F* female

[a] Studies included in 5-year overall survival (OS)

[b] Studies included in 10-year disease-free survival (DFS)

Table 2 Outcomes

Study (year)	Tumour stage before CRT (*n*)		Tumour stage after CRT (*n*)		Local recurrence						Number of patients survived disease free (5-year DFS)				Number of patients survived overall (10-year OS)			
	LE	RS	LE	RS	LE group (*n*)	RS group (*n*)	LE (*n*)	LE rate	RS (*n*)	RS rate	LE DFS (*n*)	LE DFS rate	RS DFS (*n*)	RS DFS rate	LE OS (*n*)	LE OS rate	RS OS (*n*)	RS OS rate
Bannon et al. [14] (1995)	T0, 1 T1, 5 T2, 6 T3, 2	T0/N0, 10 T1/N0, 2 T2/N0, 23 T0/N1, 1 T2/N1, 8 T3/N0, 11 T3/N1, 6 T3/N2, 4	–	–	44	65	6	13.6 %	6	9.2 %	–	–	–	–	40	90.9 %	52	80.0 %
Bonnen et al. [15] (2004)[a]	T3/N0, 25 T3/N1, 1	T3/N0, 176 T3/N1, 229	–	–	26	405	2	7.7 %	32	7.9 %	19	73.1 %	328	81.0 %	–	–	–	–
Callender et al. [16] (2010)	T3	T3	–	–	47	473	5	10.6 %	36	7.6 %	–	–	–	–	–	–	–	–
Caricato et al. [17] (2006)[a,b]	T2/N0, 5 T2/N1+, 3 T3/N0, 3 T3/N+, 14 T4/N0, 2 T4/N+, 20	T2/N0, 8	–	8	22	0	0.0 %	3	13.6 %	8	100.0 %	17	77.3 %	–	–	–	–	–
Habr-Gama et al. [18] (1998)	T1/N0, 11 T2/N0, 29 T3/N0, 38	T0/N0, 6 T1/N0, 39 T2/N0, 18 T3/N0, 14 TxNx, 1	9	78	2	22.2 %	9	11.5 %	–	–	–	–	–	–	–	–	–	–
Huh et al. [19] (2008)[a,b]	T2/N0, 1 T3/N0, 5 T3/N1, 3	–	–	–	9	64	1	11.1 %	5	7.8 %	7	77.8 %	40	62.5 %	8	88.9 %	48	75.0 %
Kundel et al. [20] (2010)[a,b]	T2/N0, 3 T3/N0, 7 T1–2/N1, 1 T3–4/N1, 3	T2/N0, 9 T3/N0, 21 T1–2/N1, 1 T3–4/N1, 6	T0/N0, 6 T1–2/N0, 6 T3/N0, 2 T1–T2/N1, 0 T3–T4/N1, 0	T0/N0, 13 T1–2/N0, 7 T3/N0, 6 T1–T2/N1, 8 T3–T4/N1, 3	14	37	0	0.0 %	3	8.1 %	14	100.0 %	33	89.2 %	14	100.0 %	36	97.3 %
Lezoche et al. [11] (2012)[a,b]	–	–	T0, 14 T1, 12	T0, 13 T1, 12	50	50	4	8.0 %	0	0.0 %	45	90.0 %	47	94.0 %	40	80.0 %	43	86.0 %

Table 2 (continued)

Study (year)	Tumour stage before CRT (*n*)		Tumour stage after CRT (*n*)		Local recurrence					Number of patients survived disease free (5-year DFS)				Number of patients survived overall (10-year OS)			
	LE	RS	LE	RS	LE group (*n*)	RS group (*n*)	LE (*n*)	LE rate	RS (*n*)	RS rate	LE DFS (*n*)	LE DFS rate	RS DFS (*n*)	RS DFS rate	LE OS (*n*)	LE OS rate	RS OS (*n*)	RS OS rate
			T2, 24	T2, 25														

LE local excision, *RS* radical surgery, *CRT* chemoradiotherapy

[a] Studies included in 5-year overall survival (OS)

[b] Studies included in 10-year disease-free survival (DFS)

1,105 patients (LE, $n=127$; RS, $n=978$). Pooled results (Fig. 5) did not demonstrate a difference in DFS between the RS and LE groups (OR 1.04, CI 0.61–1.76, $p=0.89$). Equally, the RCT [11] also did not show a difference in DFS between the RS and LE patient groups ($p=0.686$). Subgroup analysis (Fig. 6) of T3 (and greater tumours [15, 16]) showed no difference in DFS (OR 0.73, CI 0.43–1.24, $p=0.24$).

Bias

Funnel plots were used to determine potential risk of publication bias. In the studies selected for local recurrence and DFS, there were no outliers beyond the 95 % CI margins, suggesting little risk of bias.

Discussion

The findings of this systematic review and meta-analysis suggest that there are no differences in local recurrence rates, DFS and OS between patients selected for LE and RS for rectal cancer. Currently, management of rectal cancer recommends radical excision with or without neoadjuvant CRT [2]. However, a significant proportion of rectal cancers regress in size following CRT and thereby potentially become amenable for LE. This may offer a potentially safer alternative than RS, especially amongst the elderly and comorbid patient groups.

The oncological safety of LE requires consideration. The first study reporting on LE following CRT was by Marks and colleagues. This study demonstrated a 21 % local recurrence rate, although patient numbers were limited ($n=3/14$) [21]. The study population is composed largely of patients with tumours up to stage B2 (Aster Coller's staging) and those who were unfit for a major procedure. In this study, both patients with B2 cancer developed local recurrence. All three patients with local recurrence had grade II mucinous tumours. Mucinous tumours seem to be associated with aggressive behaviour and are associated with higher recurrence rates than non-mucinous colorectal cancers [22]. One of the largest series reported by Guerineri and colleagues [23], documented a local recurrence rate of 4 % in 175 patients including T2 and T3 tumours after 81 month follow-up. Conversely, Park and colleagues [24] reported no recurrences in a case series including patients staged preoperatively as T2–T3 and N0. In a review, Smith and colleagues [25] observed a local recurrence rate of 0–23 % in T2–T4 rectal cancers treated with CRT, followed by LE. Overall 5-year recurrence after RS of rectal cancer is reported as 6–8 % [4, 5]. Since the oncological outcomes suggest no difference between CRT+LE and CRT+RS, it may be argued that less invasive surgery may represent a viable alternative to RS for disease control. This may be particularly relevant to discussions with patients

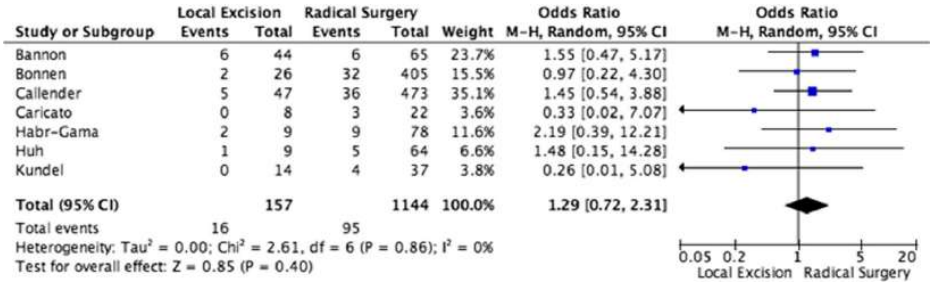

Fig. 2 Cancer recurrence across all stages

highly averse to a stoma, as well as those that represent a high perioperative risk.

Currently, LE after CRT is being mainly offered or thought to be acceptable as a palliative treatment of advanced cancers [26, 27] or in patients not wishing to undergo major surgery which may necessitate stoma formation. The studies included in this analysis is composed of patients staged preoperatively T1 to T4 and any T stage with N1. Detailed post-CRT staging was not available in most of the studies. However subgroup analysis of preoperatively staged T3 and any N tumours showed no statistical difference in the LE and RS.

After a mean follow-up of 55 months, Schell and colleagues reported survival of all 11 patients treated with CRT+LE [28]. This case series included all advanced T3 rectal cancers receiving chemoradiotherapy. However, their selection criteria for LE included tumours staged pT1 after CRT. Over a longer period of follow-up (81 months), Guerrieri and colleagues [23] reported an OS of 77 % in T3 tumours and 90 % in T2 tumours. Similar results were reported by Callender and colleagues in T3 tumours (OS 74 % over a 10-year period [16]). Rectal cancer treated with CRT and radical excision showed an overall 5-year survival of 74 % [5]. In this systematic review and meta-analysis, we considered all the results reporting at least 10 years of follow-up. There was no statistically significant difference in OS between CRT+LE and RS (83.75 vs 79 %).

DFS reflects survival in the absence of local or systemic recurrence. The German Rectal Cancer Study Group reported a DFS rate of 68 % after 5 years of follow-up in five patients after CRT+RS. Higher rates of DFS have been reported by Guerrieri and colleagues in pT2 tumours (90 %) and pT3 tumours (77 %) after median 81 months of follow-up in patients having TEM surgery following radiotherapy [23]. Our results did not demonstrate a significant difference in DFS between LE and RS post-chemoradiotherapy over 60 months of follow-up. Similar results were obtained in the subgroup analysis of T3 tumours.

All studies included in the current analysis were primarily investigating oncological outcomes. However, post-operative bowel function and the requirement of a stoma (either temporary or permanent) are important to patients, as they can significantly impact on the quality of patients' lives. After CRT+LE, Marks and colleagues reported good defecatory function in 13/14 patients and only 1/14 required a colostomy due to poor sphincter function [29]. Importantly, chemoradiotherapy treatment per se, in the absence of operative intervention, may adversely affect sphincter function [30]. Schell and colleagues have also reported the impact of low rectal cancer treatment on sphincter function [28]. Their findings demonstrated that 2/11 patients suffered sphincter-related morbidity after CRT+LE. One patient underwent successful repair for lax sphincter, and one suffered temporary faecal urgency that resolved spontaneously. A study by Do and colleagues reported good to excellent (64 %) and fair (36 %) sphincter function in patients after low anterior resection with preoperative CRT [31]. Their definition of fair sphincter function included four or more bowel movements and moderate faecal soilage with no incontinence. Low anterior resection by removing the rectal reservoir, as well as changes in pelvic nerve function, may lead to symptoms of faecal urgency, increased frequency of defecation and faecal soiling. It is conceivable that by adding CRT which itself can damage the anal canal sphincter's musculature

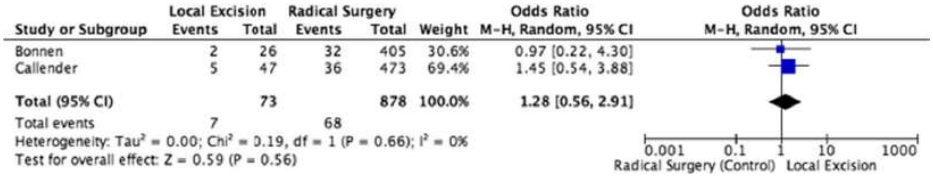

Fig. 3 Local cancer recurrence for T3 tumours only

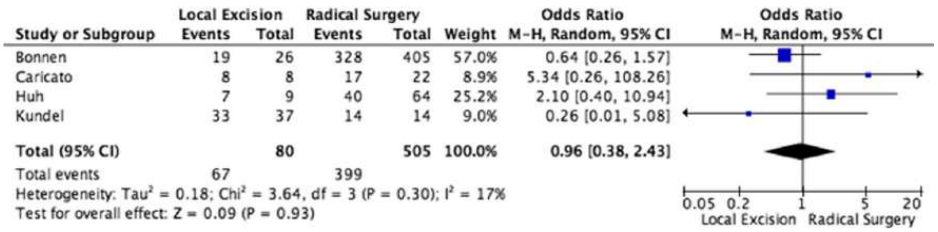

Fig. 4 Overall survival across all stages

or nerve supply may potentially compound the effect of TME surgery.

However, LE for rectal cancer is not without complications and a learning curve is associated with the technique [32]. Morbidity associated with LE may include tumour perforation—including perforation into the general peritoneal cavity in anterior higher lesions. Other short-term complications, such as rectal bleeding and suture disruption, can occur also. Suture line dehiscence may result in pelvic peritonitis. In such cases of peritonitis, 8 % (n=8/38) may require laparoscopy, washout and de-functioning ileostomy [11]. Additionally, in the study by Lezoche and colleagues, there was a 13 % (n=5/38) rate of rectal dehiscence, requiring parenteral nutrition and antibiotics. Another single surgeon series of TEMS procedures performed for both rectal adenomas and adenocarcinomas reported 13 % (33/262) morbidity, including pelvic sepsis (2.7 %), bleeding (2.7 %), rectal stenosis (1.5 %) and a mortality risk of 0.8 %. Lesions resected within 2 cm of dentate line were associated with a significantly higher risk of pelvic sepsis presumably due to lack of mesorectum in this region [33].

Long-term complications of LE surgery include rectal stenosis and poor sphincter function. One study investigating sphincter function after LE (TEM) using anorectal physiology techniques found that in the early post-operative period, there was a loss of the recto-anal inhibitory reflex, reduction in rectal maximum tolerated volumes and frequency of bowel motions. The authors reported patients experiencing episodes of temporary incontinence, possibly due to damage to the internal anal sphincter [34, 35].

Furthermore, in LE techniques, there is also an associated conversion rate to an abdominal procedure, reported as being 5.7 % (n=6/105) in the literature [32]. Reasons for such conversions include inaccessibility of lesions, large tumours and breach of the peritoneum. In this study, peritoneal breaching occurred in 9.5 % (n=10/105). In all but two patients, the peritoneum was closed transanally. Interestingly, they also noted late rectal perforation in one patient necessitating abdomen-perineal resection with permanent colostomy. While rectal perforation appears to have no effect on short- and long-term outcomes, it may prolong operative time and length of hospital stay [36].

Limitations

We aimed to investigate local recurrence and survival rates after CRT and LE by assessing the studies reporting comparative data. However, this proved difficult as there was only one RCT reporting this outcome [11]. Moreover, this RCT included T2 stage patients. All other studies were observational/cohort studies. Although these studies were assessed (Table 3) for consistency using NICE guidelines (www.nice.org.uk), in the absence of RCTs, it is difficult to interpret the results with accuracy. The follow-up period was variable, but some of the studies had accrued nearly 10 years of follow-up. The selection criteria for LE were not standardised. Bonnen and colleagues [15] in their series selected patients for LE if they refused stoma, had significant medical comorbidity or if there was complete clinical regression of tumour after CRT. Similar reasons were cited by Callender [16] and Kundel [20]. There is a lack of standard selection criteria for LE. Based on large series, it appears that for T3 cancers, 6 to 9 % of LE are performed after CRT [15, 16]. This has to be interpreted cautiously as inclusion of ypT0–T1 cancers may significantly increase the LE rate [37, 38]. Long-term adverse effects of preoperative radiotherapy

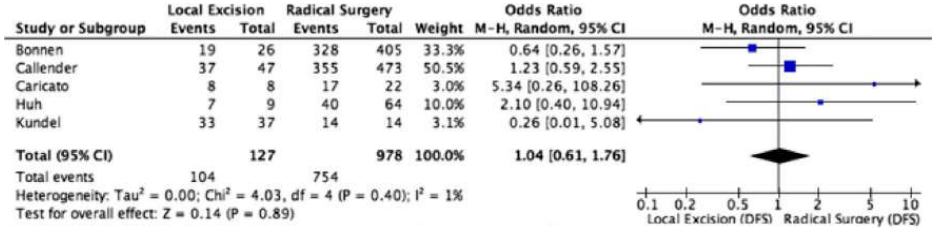

Fig. 5 Disease-free survival across all stages

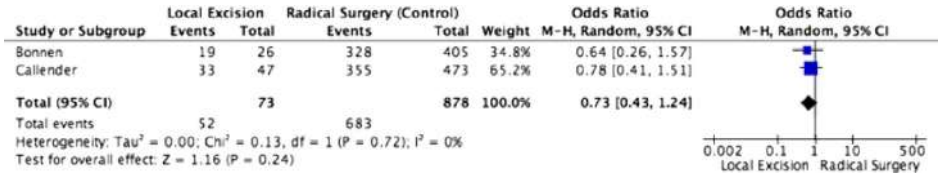

Fig. 6 DFS for T3 rectal tumours only

should not be underestimated including difficulty in rectal evacuation, incontinence, erectile dysfunction and proctitis [39, 40].

Nevertheless, this meta-analysis opens debate regarding the requirement for radical rectal excision for low rectal cancers with a good response to CRT. If lymph nodes are positive after neoadjuvant CRT, patients are optimally treated by radical excision [41] with regional lymph node clearance in line with current standards of care. However, if there is significant tumour regression and negative lymph node status, LE is a potential alternative that in this limited statistical analysis of the literature appears to offer comparable oncological outcome. This may be of particular importance in the elderly and comorbid patient for whom RS harbours significant morbidity and mortality risk.

Comparison of LE versus RS after CRT should be made for similar staging and criteria. Tumours showing regression may behave biologically less aggressively. This could be a potential pitfall when comparing outcomes of cancers after LE versus RS following CRT. There was one randomised trial by Lezoche and colleagues [11] comparing LE versus RS after CRT that showed no difference in recurrence or DFS. The technical aspect of LE may differ. In this study, the authors performed LE to include local mesorectal excision and 1 cm of normal mucosa, as well as excision of the internal sphincter in lower tumours. As mentioned above, LE can be performed by transanal full-thickness excision or minimally invasive techniques. As such, in order to meaningfully compare outcome between these surgical approaches, all these factors needs to be taken into account.

Even with ypT0 cancers, Hughes et al. reported a 17 % lymph node positivity rate in their retrospective analyses [42]. On the contrary, Kim and colleagues [43] reported only 2.2 % risk of lymph node positivity in ypT0 tumour specimens and similar results were noted by Read and colleagues [44]. This increased to 4–48 % in ypT1 to ypT4 resected specimens. As such, the authors concluded that after LE, depending on the pathological regression staging, further informed decisions can determine subsequent strategy. Habr-Gama and colleagues observed that all patients with only endoluminal recurrence had positive lymph nodes at the time of pre-CRT staging [8]. A consensus opinion of rectal cancer management, 'The Lisbon Accord', highlighted the importance of accurate post-CRT assessment of patients to investigate response. If complete response is achieved, there should be further informed discussion with the patients of options including wait and watch versus surgery, guided by digital rectal examination and high-quality MRI scanning [45].

This systematic review and meta-analysis of the current literature suggests that in the included series, there was no statistical difference in local recurrence, OS and DFS rates in patients undergoing CRT+LE versus RS for rectal cancer. The majority of studies included were however observational, and selection criteria may have varied. Large, multi-centre

Table 3 Quality assessment of the selected articles

1	Case series collected in more than one centre, i.e. multi-centre study
	NICE score (max 8)
2	Is the hypothesis/aim/objective of the study clearly described?
3	Are the inclusion and exclusion criteria (case definition) clearly reported?
4	Is there a clear definition of the outcomes reported?
5	Were data collected prospectively?
6	Is there an explicit statement that patients were recruited consecutively?
7	Are the main findings of the study clearly described?
8	Are outcomes stratified? (e.g. by disease stage, abnormal test results and patient)
Quality of assessment for case series (adapted from NICE): yes=1, no=0, score: __/8	
The combined scores of above papers used for analysis	
Bannon et.al [14]	5
Bonnen et.al [15]	4
Callender et.al [16]	5
Habr-Gama et.al [18]	4
Huh et.al [19]	4
Kundel et.al [20]	5

randomised trials are warranted to investigate this question further. LE surgery following chemoradiotherapy may represent a viable alternative to RS for patients with rectal cancer who refuse a stoma or represent a high risk for RS.

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ORIGINAL ARTICLE

Loop ileostomy in rectal cancer surgery: factors predicting reversal and stoma related morbidity

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Abstract

Purpose Loop ileostomy is performed in rectal cancer surgery to decrease the impact of anastomotic leak but it is associated with a significant complication rate. This study aimed to analyze the morbidity related to diverting ileostomy and to identify factors predictive of complications related to stoma management and reversal, as well as conversion into a permanent ileostomy.

Methods A retrospective study was conducted on 112 patients submitted to oncological rectal resection and defunctioning ileostomy in a Portuguese colorectal unit between March 2012 and March 2019.

Results Loop ileostomy was responsible for 13% of index surgery morbidity and 15% of patients' readmissions due to high output, stoma stenosis and parastomal hernia. Ileostomy was reversed in 89% cases with 7% Clavien-Dindo ≥ IIIb complications. An association was established between diabetes and higher stoma management morbidity (OR: 3.28 [95% CI: 1.039-10.426]. p = 0.041). Likewise, diabetes (OR: 0.17 [95% CI: 0.038; 6.90], p=0.015), oncological disease stage ≥ III (OR: 0.10 [95% CI: 0.005; 0.656], p=0.047) and index rectal surgery morbidity (OR: 0.23 [95% CI: 0.052; 0.955], p=0.041) were associated with less ileostomy closure. Complications of the index surgery also related to higher stoma reversal morbidity (OR: 5.11 [95% CI: 1.665; 16.346], p=0.005).

Conclusions Diabetes and complications of index rectal surgery were identified as predictive of ileostomy morbidity, closure rate and associated complications. It is essential to adjust treatment decisions to patient's morbidity risk and adopt a more selective approach concerning the use of an ileostomy.

Keywords Rectal cancer · Loop ileostomy · Morbidity · Prognostic factors

Introduction

Rectal cancer (RC) represents 44% of all colorectal neoplasia [1], and total mesorectal excision (TME) is the gold standard treatment for mid-lower rectal tumours. TME adoption has contributed to reduced local recurrence; however, the incidence rates of postoperative surgical morbidity remained almost unchanged. Anastomotic leak is the most feared complication, as it can lead to a systemic septic response if not managed promptly and may be as high as 23% [2, 3]. In order to mitigate systemic response related to anastomotic leak, there is a general trend to perform a diverting stoma in distal anastomosis and patients who received neoadjuvant radiotherapy [4]. However, this protective effect needs to be balanced against stoma morbidity [5].

Loop ileostomy complication rate is as high as 35% and can include skin problems, leakage from the stoma appliance, high output syndrome, parastomal hernia or prolapse. Stoma reversal has an overall complication rate up to 20%, postoperative ileus and surgical site infection (SSI) being the most common [6–8]. Moreover, approximately 28% of defunctioning stomas become permanent, mostly due to oncological disease progression or need for adjuvant chemotherapy [2]. Timing of stoma reversal is also a matter of debate but, accordingly to the literature, early closure does not seem to be associated with higher postoperative complications [9, 10].

The aim of this study was to analyze the morbidity related to diverting ileostomy in RC surgery and identify risk factors for complications associated with ileostomy management and reversal, as well as transformation into a permanent stoma.

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Material and methods

Study design

This was a retrospective study of all patients submitted to radical rectal resections between March 2012 and March 2019 in Hospital Beatriz Ângelo in Lisbon, followed until October 2020. Data were gathered from the electronic hospital prospective database.

Eligibility and perioperative management

Patients with adenocarcinoma of the rectum (stages T1-T4bN0-2bM0-1a, American Joint Committee on Cancer, AJCC TNM Staging Classification 8th ed., 2017), aged over 18, submitted to TME with defunctioning loop ileostomy were eligible. Patients synchronously submitted to other oncological resections were also included. Patients with resections without diverting stoma, end-colostomy or abdominoperineal resection were excluded.

Criteria for constructing a diverting loop ileostomy in the context or rectal cancer surgery were performing anterior resection with TME, partial mesorectal excision with extraperitoneal anastomosis, pouch surgery and neoadjuvant radiotherapy.

From October 2017 onwards, patients were treated according to the enhanced recovery after surgery (ERAS) protocol, systematically introduced in our institution for colorectal surgery. High output stoma was defined as the one producing more than 1L effluent/day. Stoma was prophylactically addressed with adapted antidiarrheal diet, with electrolyte mix and loperamide. The ileostomy reversal procedure was scheduled before adjuvant chemotherapy (approximately 21 days after index procedure) or after its completion. Prior to stoma reversal, the colorectal anastomosis was evaluated with digital examination, rectosigmoidoscopy and gastrografin study.

Morbidity and mortality

Morbidity related to loop ileostomy was divided in (1) morbidity of index surgery caused by ileostomy (during the first 30 postoperative days or admission for the rectal surgery), (2) morbidity associated with ileostomy management (after discharge from the index surgery admission) and (3) morbidity associated with ileostomy closure (during the first 30 postoperative days or during admission for stoma reversal).

The former 2 included dehydration due to high output, parastomal hernia, ileostomy stenosis, peri-ileostomy abscess or bleeding and hospital admissions or further surgeries resulting from stoma complications. Morbidity associated with stoma closure was categorized according to the Clavien-Dindo classification [11] and comprised SSI, anastomotic leak, ileus (absence of bowel function on postoperative day 5), gastrointestinal bleeding or small bowel obstruction (SBO). Skin problems and leakage from the stoma appliance were not included in this analysis. Regarding the index rectal surgery, colorectal anastomotic leak was defined according to the Rectal Cancer Study Group including clinical, radiological leak and perianastomotic, pelvic abscess or recto-vaginal fistula [12].

Endpoints

Primary endpoints were rate of stoma reversal, morbidity related to loop ileostomy management and to stoma closure. Secondary endpoints were clinical factors predictive of ileostomy morbidity, of complications associated with the reversal and of its transformation into a permanent stoma. Finally, the impact of time till closure on morbidity was also evaluated.

Statistical analysis

Survival analysis was performed through Kaplan–Meier (KM) statistics. Logistic regressions were used to correlate each variable with the outcomes defined: ileostomy complications, ileostomy closure and post-closure complications. Only variables with $p \leq 0.20$ in univariate logistic regression or considered clinically relevant were selected to multivariable logistic regression. Significance level was set at 0.05. Fisher's exact test and ANOVA's test were used to test the association between intersurgical time and closure morbidity. No logistic regression analysis had evidence of poor fit (Hosmer and Lemeshow Goodness of Fit). Data was analyzed with R (version 4.0.2, 2020-06-22, "Taking Off Again").

Results

Demographic characteristics

During the study period, a total of 220 consecutive RC patients were submitted to surgical treatment of which 112 were included in this analysis (Fig. 1). Demographic and clinical parameters are summarized in Table 1.

Staging, neoadjuvant therapy and index surgery

Of the 112 patients, 111 (99%) were treated for RC and one (1%) for premalignant disease (endoscopically non-resectable adenoma with high-grade dysplasia) (Table 2). At diagnosis, 66 (60%) patients presented disease stage III with a median carcinoembryonic antigen (CEA) of 1.7 ng/mL (0.8–3.2). Eighty-one (72%) patients had some type of neoadjuvant treatment while 31 (28%) underwent direct resection. Twenty-one (19%) patients underwent a transanal total mesorectal excision (TaTME) and 91 (81%) an anterior resection (AR), 10 with synchronous liver metastasectomy or

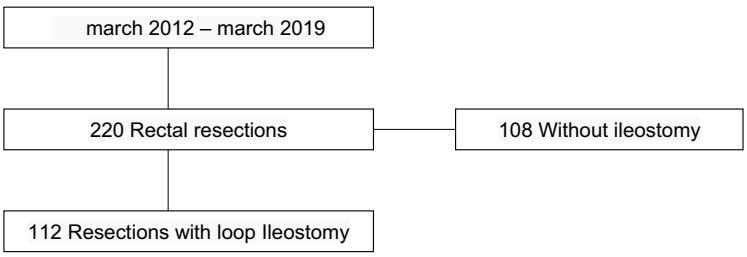

Fig. 1 Volume of radical resections for rectal cancer. Unit's volume of radical resections (non-exenterative) for rectal cancer during the elected period of the study

colectomy. Laparoscopic approach was used in 73 (65%) patients with 12 (11%) cases requiring conversion.

There were 53 (47%) postoperative complications of the rectal surgery, 22 (20%) Clavien-Dindo≥ IIIb.

Overall anastomotic insufficiency rate was 11% (12/112), related to 3 peri-anastomotic collections treated with antibiotic (Clavien II), 1 leak treated with endo-SPONGE® (Clavien IIIa) and 8 that needed surgical re-exploration (Clavien IIIb).

Seventy-two (64%) patients underwent adjuvant chemotherapy, 67 (93%) before ileostomy reversal (Table 2).

Ileostomy reversal

From the initial 112 patients, 2 died in the index surgery postoperative period, 1 with the ileostomy closed and the other without. So, from the 111 patients eligible for closure, 99 (89%) underwent ileostomy reversal with a median time interval from primary procedure of 8.4 (5.9–11.9) months. The majority (60%) of patients had a stapled side-to-side anastomosis. Median duration of reversal procedure was 80 (60–100) min; skin closure technique was a purse-string in 41 (45%) patients and primary closure in 36 (40%) (Table 3).

Loop ileostomy as permanent stoma

Twelve (11%) patients did not undergo reversal of the stoma, of which six presented with disease stage III and five with stage IV at diagnosis (Table 3). Reasons for not closing the stoma were disease progression in six patients, two colorectal anastomotic strictures, one metachronous second cancer, two patient refusals and one colorectal anastomotic leak

Ileostomy-related morbidity

Loop ileostomy was responsible for seven (13%) of the 53 morbidity cases of the index RC surgery, namely, one high output stoma, one peristomal bleeding, two peristomal abscesses, one ileostomy stenosis with obstruction and two strangulated parastomal hernias that prompted urgent surgical exploration (Table 4).

After discharge from the index surgery, of the 110 patients with loop ileostomies, 16 (15%) presented with complications that required 20 hospital readmissions: 18 cases of dehydration due to high output, one obstruction secondary to ileostomy stenosis and one strangulated parastomal hernia. During follow-up, six (5%) patients also developed paraileostomy hernias but these did not warrant surgical intervention or readmission (Table 4).

Likewise, 24 (24%) patients had complications of ileostomy closure, four (4%) of which were Clavien-Dindo IIIb: one anastomotic leak, one small bowel obstruction, one iatrogenic enterotomy and one small bowel ischemia. There were three (3%) deaths in the postoperative period, 2 due to anastomotic leak and one to pneumonia. Reoperation rate was 6.1% (Table 4).

Factors predictive of ileostomy morbidity

In order to test the association between clinical factors and ileostomy morbidity, logistic regression analysis was

Table 1 Patient clinical parameters

Clinical parameters		Patients (n = 112)
Gender, n (%)	Female	38 (33.9)
	Male	74 (66.1)
Age, median		67 (60–74)
Obesity, n (%)	No	99 (88.4%)
	Yes	13 (11.6%)
Respiratory comorbidities, n (%)	No	98 (87.5%)
	Yes	14 (12.5%)
Cardiac comorbidities, n (%)	No	81 (72.3%)
	Yes	31 (27.7%)
HBP, n (%)	No	48 (42.9%)
	Yes	64 (57.1%)
Diabetes, n (%)	No	85 (75.9%)
	Yes	27 (24.1%)
ASA score, n (%)	I	1 (0.9%)
	II	69 (61.6%)
	III	40 (35.7%)
	IV	2 (1.8%)

Obesity: body mass index over 30; Cardiac comorbidities: disarrhythmias (atrial fibrillation, need of pacemaker, left or right cardiac blockage), coronary disease, cardiac insufficiency, aortic or mitral stenosis or insufficiency and past medical history of stroke or myocardial infarction; respiratory comorbidities: chronic obstructive pulmonary disease (COPD), sleep apnoea, or chronic pulmonary embolism; *HBP* high blood pressure; *ASA* American Society of Anaesthesiologists

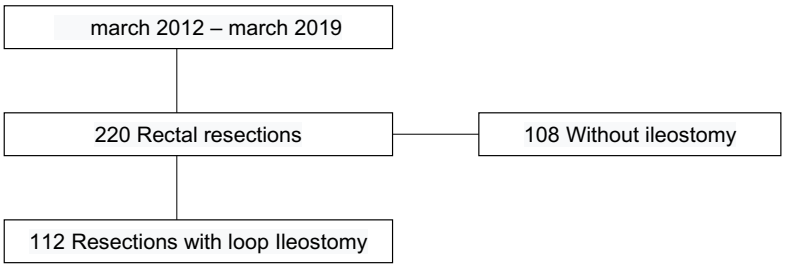

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Table 3 Ileostomy reversal

Clinical parameters		Patients (n = 111)
Ileostomy reversal, n (%)	No	12 (10.8%)
	Yes	99 (89.2%)
Time till ileostomy reversal, months		8.4 (5.9–11.9)
Pre op total seric protein, median		6.7 (6.2–7.2)
Pre op seric albumin, median		4.3 (3.8–4.4)
Pre op CRP, mg/dL, median		0.71 (0.2–1.2)
Ileostomy closure time, min		80 (60–100)
Surgical anastomosis, *n* (%)	Side-to-side handsewn	4 (4.1%)
	Side to side mechanical	58 (59.8%)
	End to end manual	35 (36.1%)
	NA or ND	15
Surgical skin closure, *n* (%)	Purse-string	41 (45.1%)
	Primary closure	36 (39.6%)
	Primary closure over drain	14 (15.4%)
	NA or ND	21
Time till diet tolerance, days (median)		2.5 (1–7)
Time till bowel transit, days (median)		3 (2–7)
LOS, days (median)		5 (4–8)

One patient that died after index surgery was not eligible for ileostomy closure rate and was not included in this analysis . *CRP* seric C reactive protein; *LOS* length of hospital stay; *NA* not applicable; *ND* not discriminated

Discussion

Loop ileostomy is performed in rectal surgery to decrease morbidity and mortality associated with dehiscence of colorectal anastomosis [13]. The decision to create this defunctioning stoma is influenced by anastomosis site and pre-/intraoperative risk factors for leak [14]. If some procedures have a dehiscence risk that warrants routine diversion (11% for ultralow/ coloanal and 13% for ileal pouch anal anastomosis), others have variable leak rates that question constructing a defunctioning stoma (3–23% for anterior resection) [13, 15, 16]. Overall, leak rate is reported from 5–23% and is associated with considerable morbidity, mortality, higher cancer recurrence, diminished bowel function and quality of life [13, 17, 18].

Although ileostomy does reduce these poor consequences of a dehiscence, 85–90% of patients do not endure this problem, do not benefit from a stoma and are unnecessarily exposed to its potential morbidity. Proponents of a diverting ileostomy claim a minor negative impact derived from the stoma [19] but arguments for omitting it rely precisely on avoiding associated morbidity, evading intestinal atrophy with immediate use of anal sphincter and the need for only a single hospital admission.

The ileostomy morbidity relates not just to the reversal, often considered a "minor" procedure, but also to the management of the stoma itself. In fact, overall morbidity is reported as high as 35% [20–22] with skin irritation, retraction, prolapse, dehydration and electrolyte disturbance from high output that often lead to hospital readmissions [2, 23–25]. Also, wound infection is reported as high as 18.3%, small bowel obstruction as 15%, enterocutaneous fistula in 0.5–7%, anastomotic dehiscence up to 8% and parastomal hernia up to 12% [26]. Subsequent laparotomy can be needed (3.7%) to close the stoma in the presence of adhesions, obstruction or hernia. Moreover, having an ileostomy significantly impacts on the quality of life [18, 27] and a meaningful proportion of the so-called "transient" stomas are never reversed [19, 28]. Finally, one always has to consider a mortality risk [21].

Currently we still lack precise data on rates of major morbidity associated with ileostomy management and reversal. Likewise, identification of risk factors for these complications could improve patient selection allowing individualized decisions on endorsing or avoiding diversion. In such a controversial setting, this study aimed to evaluate, in a colorectal unit, the stoma closure and morbidity rates associated with ileostomy performed in RC surgery. We intended to analyze modifiable and non-modifiable risk factors predictive of morbidity and stoma closure and investigate whether surgical techniques play a role, in a context of disagreement about the optimal anastomotic procedure.

In the present study, we observed 22 (20%) cases of complications of ileostomy maintenance, with 16 (15%) patients needing readmission, mainly due to dehydration for high output stoma. Regarding risk factors for this morbidity, univariate analysis identified diabetes, age and HBP as associated with

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Table 4 Ileostomy-associated morbidity

Clinical parameters		
Morbidity of index surgery caused by ileostomy, *n* (%)		7/ 112 (6.3)
Clavien-Dindo, *n* (%)	Grade II	5 (4.5)
	Grade III b	2 (1.8)
Type	High output, dehydration	1
	Ileostomy stenosis	1
	Parastomal hernia	2
	Peri-ileostomy bleeding	1
	Peri-ileostomy abscess	2
Morbidity of ileostomy management, *n* (%)		22/ 110 (20)
Type	High output, dehydration	14
	Ileostomy stenosis	1
	Parastomal hernia	7
Readmissions		20 (18.2)
Morbidity of ileostomy closure		24/ 99 (24)
Clavien-Dindo, *n* (%)	Grade I	3 (3.0)
	Grade II	12 (12.0)
	Grade IIIa	2 (2.0)
	Grade IIIb	4 (4.0)
	Grade V	3 (3.0)
Type	SSI	10
	Anastomotic leak	3
	UTI	2
	Ileus	2
	SBO	2
	Gastrointestinal bleeding	1
	Iatrogenic enterotomy	1
	Pseudomembranous colitis	1
	Pneumonia	1
	Fever	1
Reoperation, *n* (%)		6 (6.1)

Two patients that died after index surgery were not included in the morbidity of ileostomy management data; Morbidity of ileostomy closure included data on the 99 patients that closed their stoma; morbidity according to Clavien-Dindo's classification; *SSI* surgical site infection; *UTI* urinary tract infection; *SBO* small bowel obstruction

complications. Multivariable analysis, however, validated this association only for diabetes with odds of complications 3.28 times greater in individuals with this disease when compared with patients without (OR: 3.28 [95% CI: 1.039; 10.426], p = 0.041).

We report 89% ileostomy closure rate with a median time to reversal of 8.4 (5.9–11.9) months, higher than some series [4, 22]. This relates to the fact that, in our institution, adjuvant chemotherapy is mainly performed prior to stoma closure, delaying the procedure. In multivariable analysis, there was a statistically significant association between stoma closure and gender (OR: 0.11 [95% CI: 0.005; 0.673], p=0.049), diabetes (OR: 0.17 [95% CI: 0.038; 0.690], p=0.015), stage (OR: 0.10 [95% CI: 0.005; 0.656], p=0.047) and morbidity of the index surgery (OR: 0.23 [95% CI: 0.052; 0.955], p=0.041). Overall, ileostomy closure was decreased in females, patients with diabetes, higher clinical stages and complications of the index rectal surgery. It is interesting to note that, in our series, 12 (11%) patients did not undergo stoma reversal mainly related to oncological disease progression. Although treated with a curative intent, these patients had locally advanced and metastatic disease (III and IV) that seems to negatively influence closure. Our results are in concordance with previous reports that show that an important part of the pretended "temporary" stomas are never reversed [19, 28]. In this setting, one might question if, in patients with more advanced disease, the option should be for a non-restorative procedure from the outset. Late colorectal anastomotic strictures or leak, metachronous second cancer and patient refusal were also reasons for non-closing. Interestingly, although delaying reversal, adjuvant chemotherapy did not impact on the rate of stoma closure.

Table 5 Factors predictive of ileostomy morbidity

Variables	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Female	0.83 (0.283; 2.637)	0.745		
Age	1.07 (1.009; 1.149)	0.034	1.07 (1.004; 1.612)	0.050
Obesity	1.10 (0.160; 4.705)	0.904		
Respiratory comorbidities	1.78 (0.368; 6.676)	0.419		
Cardiac comorbidities	1.70 (0.533; 5.088)	0.347		
Diabetes	4.05 (1.335; 12.428)	0.013	3.28 (1.039; 10.426)	0.041
HBP	3.82 (1.144; 17.452)	0.046		
ASA				
I+II				
III+IV	2.45 (0.841; 7.441)	0.101		
CEA (pre-treatment)	0.982 (0.873; 1.005)	0.589		
Stage (AJCC)				
I+II				
III+IV	0.92 (0.304; 3.139)	0.886		
Neoadjuvant therapy				
Direct surgery	0.56 (0.122; 1.904)	0.394	0.40 (0.087; 1.453)	0.196
CT/LCCRT/SCRT				
Tumour location				
High rectum				
Mid rectum	0.17 (0.025; 0.733)	0.032		
Low rectum	0.44 (0.123; 1.431)	0.183		
Index surgical procedure				
AR				
TaTME	1.00 (0.285;4.686)	0.999		
Index surgical approach				
Laparotomy				
Laparoscopy	2.02 (0.681; 6.830)	0.221		
Index surgery morbidity	1.52 (0.524; 4.570)	0.442		
Index surgery morbidity				
Clavien-Dindo 0-IIIa				
Clavien-Dindo IIIb-V	0.94 (0.200; 3.268)	0.923		

Multiple logistic regression analysis using ileostomy morbidity as dependent variable. *OR* odds ratio; *CI* confidence interval; *HBP* high blood pressure; *ASA* American Society of Anaesthesiologists; *CEA* carcinoembryonic antigen; *AJCC* American Joint Committee on Cancer TNM Staging Classification for Rectal Cancer 8th ed., 2017; *CT* chemotherapy; *LCCRT* long course chemoradiotherapy; *SCRT* short course chemoradiotherapy; *AR* anterior resection; *TaTME* transanal total mesorectal excision; *Clavien-Dindo* Clavien Dindo classification of surgical morbidity. Hosmer and Lemeshow Goodness of Fit (p=0.431) indicates no evidence of poor fit

Concerning ileostomy closure morbidity, we observed 24% of complications, 7% equal to or over Clavien-Dindo IIIb with 6% reoperation rate. Other groups reported similar results including a meta-analysis that reviewed 6107 patients in 48 studies that showed 17.3% ileostomy closure complications and 3.7% reoperation rate [21, 22, 29–31]. In our study, a significant association was identified between complications of the index rectal surgery and complications of ileostomy closure (OR: 5.11 [95% CI: 1.665; 16.346], p=0.005). In fact, patients with index surgery complications graded higher than Clavien-Dindo IIIa had increased odds of ileostomy closure problems. This could be explained by the fact that, when abdominal re-exploration is needed, it is often performed by laparotomy, increasing adhesions that difficult subsequent ileostomy closure. Intriguingly, morbidity of ileostomy closure was not influenced by preoperative CRP, total protein and albumin.

There are other controversies regarding ileostomy in RC surgery, one of them is timing of ileostomy closure. Previous authors have identified prolonged intersurgery period as associated with an increase in complications of stoma closure [4, 32, 33]. On the contrary, it has also been showed that early closure resulted in more postoperative complications than late one [34]. Others, including a recent meta-analysis, however,

Table 6 Factors predictive of ileostomy closure

Variables	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Female	0.14 (0.008; 0.753)	0.064	0.11 (0.005; 0.673)	0.049
Age	1.01 (0.956; 1.072)	0.647		
Obesity	1.66 (0.285; 31.491)	0.642		
Cardiac comorbidities	2.28 (0.566; 15.317)	0.303		
Diabetes	0.31 (0.094; 1.068)	0.057	0.17 (0.038; 0.690)	0.015
HBP	1.16 (0.351; 3.750)	0.798		
ASA				
I+II				
III+IV	0.95 (0.296; 3.360)	0.939		
CEA (pre-treatment)	1.00 (NA; 1.00)	0.319		
Stage (AJCC)				
I+II				
III+IV	0.17 (0.009;0.931)	0.098	0.10 (0.005; 0.656)	0.047
Tumour location				
High rectum				
Mid rectum	0.47 (0.093; 1.943)	0.314		
Low rectum	0.80 (0.147; 3.869)	0.775		
Surgical procedure				
AR				
TaTME	0.77 (0.112; 3.167)	0.741		
Surgical approach				
Laparotomy				
Laparoscopy	0.72 (0.205; 2.312)	0.587		
Index surgery morbidity	0.52 (0.148;1.672)	0.281		
Index surgery morbidity				
Clavien-Dindo 0–IIIa				
Clavien-Dindo IIIb–V	0.33 (0.098; 1.210)	0.079	0.23 (0.052; 0.955)	0.041
Adjuvant CT	0.91 (0.213;6.254)	0.904		
Ileostomy morbidity	1.51 (0.454;4.915)	0.488		
Number of readmissions for ileostomy morbidity	0.17 (0.009;1.594)	0.139		

Multiple logistic regression analysis using ileostomy closure as dependent variable. *OR* odds ratio; *CI* confidence interval; *HBP* high blood pressure; *ASA* American Society of Anaesthesiologists; *CEA* carcinoembrionic antigen; *AJCC* American Joint Committee on Cancer TNM Staging Classification for Rectal Cancer 8th ed., 2017; *AR* anterior resection; *TaTME* transanal total mesorectal excision Hosmer and Lemeshow Goodness of Fit (p=0.992), indicates no evidence of poor fit

reported no significant difference in the postoperative morbidity rate, anastomotic leak, small bowel obstruction, bleeding or ileus between early versus late ileostomy reversal. SSI was the only parameter significantly elevated after early closure in comparison with late one [9, 10, 35–38]. In our study, time to closure did not impact on the stoma morbidity, closure or related complications. Finally, there was no impact of the anastomotic technique and type of approach (laparoscopic or open) of the index surgery on closure complications [39, 40].

This study's main limitation is the small sample size and its retrospective nature. However, it is based exclusively on patients treated for RC with a long follow-up, offering a perception of the outcomes following ileostomy creation in a real-life cohort. It is interesting to note that, in a series with 76% of diabetic patients, *diabetes mellitus* predicted an increase in complications of ileostomy management, inferior closure rate and increased morbidity of stoma closure. Additionally, morbidity of the initial rectal surgery had a significant impact on the rates of ileostomy closure and associated morbidity.

The difficulty is that many of the patients at high risk of ileostomy complications are also at high threat of anastomotic leak. When deciding over diverting an anastomosis, the influence of predictive factors must be taken into account. Modifiable risk factors like *diabetes mellitus* can be improved

Table 7 Factors predictive of ileostomy closure morbidity

Variables	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Female	0.69 (0.274; 1.758)	0.429		
Age	1.00 (0.955; 1.047)	0.970		
Obesity	0.24 (0.013; 1.329)	0.181		
Respiratory comorbidities	1.22 (0.309; 4.080)	0.758		
Cardiac comorbidities	1.19 (0.429; 3.113)	0.731		
Diabetes	2.21 (0.766; 6.165)	0.132	2.57 (0.846; 7.693)	0.090
HBP	0.48 (0.187; 1.193)	0.116		
ASA I, II	0.92 (0.349; 2.340)	0.870		
Pre-treatment CEA	1.03 (1.002; 1.079)	0.107		
Stage (AJCC)				
III+IV	0.59 (0.229; 1.562)	0.281		
Neoadjuvant therapy				
Direct surgery	1.33 (0.497; 3.426)	0.560		
CT/LCCRT/SCRT				
Surgical procedure				
AR				
TaTME	0.93 (0.312; 3.177)	0.905		
Surgical approach				
Laparotomy				
Laparoscopy	0.92 (0.370; 2.305)	0.859		
Index surgery morbidity	1.77 (0.710; 4.487)	0.223		
Index surgery morbidity				
Clavien-Dindo 0-IIIa				
Clavien-Dindo IIIb–V	4.64 (1.550; 14.287)	0.006	5.11 (1.665; 16.346)	0.005
Adjuvant CT	0.46 (0.179; 1.167)	0.100		
Adjuvant CT prior closure	7.50 (1.111; 62.875)	0.039		
Ileostomy morbidity	1.22 (0.309; 4.080)	0.758		
Number of readmissions for ileostomy morbidity	1.33 (0.051; 20.212)	0.837		
Pre op total seric protein	1.19 (0.550; 2.648)	0.658		
Pre op seric albumin	0.84 (0.246; 2.976)	0.771		
Pre op seric CRP	1.08 (0.907; 1.308	0.389		
Ileostomy closure time	1.01 (1.002; 1.025)	0.026		
Anastomosis				
S-S handsewn				
S-S mechanical	0.95 (0.112; 20.148)	0.969		
E-E handsewn	1.20 (0.134; 25.870)	0.881		
Skin closure				
Purse-string				
Primary closure	1.05 (0.379; 2.879)	0.926		
Time to diet tolerance	1.01 (0.864; 1.174)	0.913		
Time to bowel transit	1.01 (0.843; 1.203)	0.928		
Time to stoma closure	1.02 (0.941; 1.109)	0.587		

Multiple logistic regression analysis using ileostomy closure as dependent variable. *OR* odds ratio; *CI* confidence interval; *HBP* high blood pressure; *ASA* American Society of Anaesthesiologists; *CEA* carcinoembrionic antigen; *AJCC* American Joint Committee on Cancer TNM Staging Classification for Rectal Cancer 8th ed., 2017; *CT* chemotherapy; *LCCRT* long course chemoradiotherapy; *SCRT* short course chemoradiotherapy; *AR* anterior resection; *TaTME* transanal total mesorectal excision; *CRP* C reactive protein; *S-S* side-to-side anastomosis technique; *E-E* end-to-end anastomosis technique. Hosmer and Lemeshow Goodness of Fit (p=0.992), indicates no evidence of poor fit

prior to constructing a derivative stoma. In the particular cases of very advanced disease, considering the negative impact on ileostomy closure rate, one should consider a non-restorative procedure. Having said this, if oncologically feasible, the goal is to preserve sphincter function aiming to reduce the rate of unnecessary ileostomies.

Conclusions

This study identified *diabetes mellitus* and morbidity of the index rectal surgery as factors predictive of ileostomy morbidity, reversal and related complications. In order to decrease morbidity related to loop ileostomy, preoperative optimization of *diabetes* since rectal cancer diagnosis should be routinely implemented. Also, we must acknowledge the importance of optimizing the short-term results of the primary surgery in the ileostomy-related outcomes.

It is essential to adjust treatment decisions to patient's predicted morbidity risk and adopt a more selective approach concerning the use of a defunctioning ileostomy, especially for patients in which the risk of having a stoma may offset potential advantages.

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Availability of data and material The datasets analyzed during the current study are available in the Hospital Beatriz Ângelo informatics hospital database (Soarien), available from the corresponding author on reasonable request.

Code availability Not applicable.

Authors' contributions Susana Ourô: study conception and design, acquisition, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article; Marisa P Ferreira: study conception and design, acquisition, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article; Diogo Albergaria: critical revision of the article for important intellectual content, final approval of the article; Rui Maio: critical revision of the article for important intellectual content, final approval of the article.

Declarations

Ethics approval/Consent to participate/Consent for publication The present study was approved by the Ethics Committee and Institutional Review Board of Hospital Beatriz Ângelo with no formal Informed consent required due to its methodology and anonymity. The study protocol was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

Competing interests The authors declare no competing interests.

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Incidence and Risk Factors for Anastomotic Failure in 1594 Patients Treated by Transanal Total Mesorectal Excision

Incidence and Risk Factors for Anastomotic Failure in 1594 Patients Treated by Transanal Total Mesorectal Excision

Results From the International TaTME Registry

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Objective: To determine the incidence of anastomotic-related morbidity following Transanal Total Mesorectal Excision (TaTME) and identify independent risk factors for failure.

Background: Anastomotic leak and its sequelae are dreaded complications following gastrointestinal surgery. TaTME is a recent technique for rectal resection, which includes novel anastomotic techniques.

Methods: Prospective study of consecutive reconstructed TaTME cases recorded over 30 months in 107 surgical centers across 29 countries. Primary endpoint was ''anastomotic failure,'' defined as a composite endpoint of early or delayed leak, pelvic abscess, anastomotic fistula, chronic sinus, or anastomotic stricture. Multivariate regression analysis performed identifying independent risk factors of anastomotic failure and an observed risk score developed.

Results: One thousand five hundred ninety-four cases with anastomotic reconstruction were analyzed; 96.6% performed for cancer. Median anastomotic height from anal verge was 3.0 ± 2.0 cm with stapled techniques accounting for 66.0%. The overall anastomotic failure rate was 15.7%. This included early (7.8%) and delayed leak (2.0%), pelvic abscess (4.7%), anastomotic fistula (0.8%), chronic sinus (0.9%), and anastomotic stricture in 3.6% of cases. Independent risk factors of anastomotic failure were: male sex, obesity, smoking, diabetes mellitus, tumors >25 mm, excessive intraoperative blood loss, manual anastomosis, and prolonged perineal operative time. A scoring system for preoperative risk factors was associated with observed rates of anastomotic failure between 6.3% to 50% based on the cumulative score.

Conclusions: Large tumors in obese, diabetic male patients who smoke have the highest risk of anastomotic failure. Acknowledging such risk factors can guide appropriate consent and clinical decision-making that may reduce anastomotic-related morbidity.

Keywords: anastomotic failure, incidence, rectal surgery, risk factors, total mesorectal excision, transanal

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Anastomotic leakage (AL) is a common and potentially devastating complication of a colorectal anastomosis and can result in severe morbidity and mortality, as well as long-term anorectal dysfunction.[1] Additionally, AL has been reported to increase the risk of local cancer recurrence,[2] with reduction in overall and disease-free survival.[3–5] AL can markedly impair a patient's quality of life and is detrimental to the doctor–patient relationship,[6] particularly as AL can result in prolonged sequelae including anastomotic fistulae, chronic sinuses, and anastomotic strictures. The reported incidence of AL after colorectal surgery is between 2 and 24%,[7–10] with the highest rates after low anterior resection.[11,12] The clinical manifestations, and severity, of AL encompass a broad spectrum of symptoms, and signs, from minor symptoms, to major life-threatening events.

As a consequence of technical developments, particularly stapling instruments, but also minimal access techniques, in combination with widespread adoption of total mesorectal excision as the standard treatment for rectal cancer, the rate of sphincter-preserving surgery with low anastomoses has significantly risen. The reduction in abdomino-perineal excision rates, with an increase in low anastomoses, has led to an increased overall leakage rate in patients with rectal cancer.[13] Technical drawbacks of minimal access intracorporal anastomosis include the lack of direct tactile sensation, inadequate exposure, and a suboptimal cutting angle of the endo-linear stapler. Crossing staple lines by repeated firings, or incorrect staple height in relation to tissue thickness increases the risk of AL, especially when 3 or more linear staple firings are needed.[14,15] Transanal total mesorectal excision (TaTME) is the latest advanced surgical access technique for pelvic dissection and facilitates different anastomotic techniques without the need for transabdominal rectal transection, particularly in a narrow pelvis. The standard TaTME technique incorporates an open rectal stump with continuity restored by a coloanal handsewn or double purse-string stapled anastomosis.[16] As TaTME adoption increases, careful monitoring and review of outcomes is crucial. Identification of risk factors for AL and overall anastomotic failure may guide preoperative optimization and intraoperative surgical decision-making, adopting measures to reduce risk and consequences of AL, such as selective defunctioning stomas. This is even more important when a novel anastomotic technique is being implemented into clinical practice.

The primary aim of this study was to report ''anastomotic failure'' rates and incidence of anastomosis-related morbidity in

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Ethical Approval: Ethical approval for the taTME registry and publication of its results was obtained from the UK Health Research Authority (REC reference 15/LO/0499, IRAS project ID 156930).

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patients following TaTME surgical procedures recorded on the international TaTME registry. The secondary aim was to identify potential risk factors associated with anastomotic failure.

METHODS

Study Design

Cases recorded on the international TaTME registry[17] between July 2014 and December 2016 by 107 surgical centers in 29 different countries (Appendix 1) were analyzed. The registry is a secure online database open to all international surgeons performing TaTME, as previously described.[18] All contributing surgeons were invited via emails to update their records with 2 subsequent reminders to obtain up-to-date data and minimize missing fields. Contributing surgeons were contacted individually to clarify any unexpected or ambiguous data. The primary endpoint of the study was ''anastomotic failure'' rate, defined as the overall incidence of anastomotic-related morbidity, including early and late AL, pelvic abscess, anastomotic-related fistula, chronic sinus, and persistent anastomotic stricture after primary rectal resection. ''Early'' anastomotic leak was defined as a symptomatic leak diagnosed and managed within 30 days of the primary resection. Anastomotic leaks were classified according to the ''International Study Group of Rectal Cancer'' definition and severity grading system (Appendix 2).[19]

Statistical Analysis

All categorical data are presented as number of cases and percentages, while continuous data are shown as either mean ± standstandard deviation (range) or median with range. Categorical variables were compared by the Pearson Chi^2 test, and continuous variables by the 2-sample *t* test or Mann–Whitney *U* test where appropriate. Risk factors were divided into patient, tumor-related factors, and technical intraoperative factors. Continuous variables were dichotomized using the median or the value at which a significant change occurred as a cut-off point. Variables that achieved a *P* value of $\leq 0{\cdot}100$ on univariate analysis were selected for the multivariate analysis to identify independent predictors of anastomotic failure and early AL. Median and mean imputation was used to adjust for missing values where appropriate and first-order interactions tested in the multivariate model. A *P* value $<0{\cdot}05$ was considered statistically significant and odds ratios (OR) and their 95% confidence intervals (CI) are reported. The β coefficients (log odds ratios) derived from the multivariate analysis were used as weights in the derivation of the anastomotic failure observed risk score. Multilevel logistic regression model was used to adjust for possible clustering of anastomotic failure within centers. The Statistical Package for Social Sciences (SPSS) of IBM Statistics, version 24, was used for the analysis.

RESULTS

A total of 1836 cases were recorded on the TaTME registry over a 30-month period. The indication for surgery was rectal cancer in 1663 (90.6%) patients and benign pathology in 173 (9.4%). Overall, 1594 of 1836 (86.8%) cases had an anastomosis and will be the focus of the results presented in this paper. Of the remaining 242 nonrestorative procedures, 236 were planned as such, leaving 6 (0.4%) cases in which the anastomosis was abandoned (Supplementary Table 1, http://links.lww.com/SLA/B360).

Patient and Tumor Characteristics

Table 1 outlines patient and tumor characteristics. The majority of registered cases were male patients with a median (range) age of 65 (19–93) years and median (range) body mass index (BMI) of 26.0 (15.6–44.2) kg/m^2. In total 275 patients (17.3%) had previous unrelated abdominal surgery, including 21 (1.3%) prior prostatectomy. Twelve patients (0.8%) had received pelvic radiotherapy prior to diagnosis of rectal cancer. The indication for surgery was rectal cancer in 1540 (96.6%) of reconstructed cases with a median tumor height from anorectal junction on staging MRI of 4.0 (0–14) cm. Radiological cancer staging was reported as stage 0, I, II, III, and IV in 17 (1.2%), 267 (19.5%), 287 (20.9%), 689 (50.2%), and 112 (8.2%) cases respectively. Preoperative involvement of the circumferential resection margin was seen on 274 (23.4%) staging MRI scans and 895 (56.1) patients received neoadjuvant therapy; the majority as long course chemoradiotherapy.

TABLE 1. Patient and Tumor Characteristics

Factor	TaTME Registry Data Results
Category	**Total: 1594 Cases**
Sex, n (%)	
Male	1080 (67.8)
Female	514 (32.2)
Age in years, mean ± SD (range)	63.7 ± 12.4 (19–93)
ASA score, n (%)	
I + II	1271 (80.7)
III + IV	303 (19.3)
Missing	*20 (1.3)*
BMI in kg/m^2, mean ± SD (range)	26.3 ± 4.4 (15.6–44.2)
Smoking, n (%)	
Smoker	230 (14.4)
Nonsmoker	1364 (85.6)
Presence of comorbidities, n (%)	
Diabetes mellitus	178 (11.2)
Ischemic heart disease	222 (13.9)
Active inflammatory bowel disease	30 (1.9)
Steroid use at the time of surgery	16 (1.0)
Previous unrelated abdominal surgery, n (%)	275 (17.3)
Clinical tumor height from anal verge on rigid sigmoidoscopy in cm, median (range)	6.0 (0–17)
Tumor height from anorectal junction on MRI in cm, median (range)	4.0 (0–14)
Preoperative MRI staging, n (%)	
≥mrT3	930 (69.0)
mrN+	764 (57.3)
Preoperative CRM involvement on MRI*, n (%)	274 (23.4)
Received neoadjuvant therapy, n (%)	895 (56.1)
TRG response post neoadjuvant therapy, n (%)	
mrTRG 1 and 2 (no or small residual tumor)	446 (52.0)
mrTRG 3 (mixed fibrosis and tumor)	220 (25.6)
mrTRG 4 and 5 (mainly or only tumor)	192 (22.4)

*CRM involvement on MRI is defined as involved if the distance of tumor or malignant lymph node to the mesorectal fascia was less than 1 mm on MRI. Percentages for missing values use the total number of cancer cases as the denominator (ie, 1594). Percentages for the variables are calculated out of the total number of actual results available excluding the missing values.

SD indicates standard deviation; ASA, American Society of Anesthesiologists; MRI, magnetic resonance imaging; CRM, circumferential resection margin; N+, positive nodal status (N1 or N2); TRG, tumor regression grading on MRI.

Intraoperative Details

Operative details are summarized in Table 2, showing that the commonest operation performed was a low anterior resection in 89%, with synchronous operating by 2 teams in 41.7%. The abdominal phase was performed laparoscopically in 1350 (86.3%); with SILS, open surgery, and robotic approaches in 179 (11.4%), 26 (1.7%), and 10 (0.6%), respectively. The recorded estimated blood loss was 0 to 99 mL in 42.3% and 100 to 499 mL in 21.1%. In 32 (2.1%) blood

TABLE 2. Operative Details

Operative Characteristics	**TaTME Registry Data Results**
Factor	**Total = 1594 Cases**
Category	**n (%)**
Indication	
Benign	54 (3.4)
Cancer	1540 (96.6)
Operations performed	
Cancer cases:	
High anterior resection	122 (7.9)
Low anterior resection	1411 (91.6)
Total and subtotal colectomies	7 (0.5)
Benign cases:	
Low anterior resection	9 (16.6)
Proctectomy (close rectal) + IPAA	6 (11.1)
Proctectomy (TME plane) + IPAA	37 (68.5)
Completion proctectomy	1 (1.9)
Total colectomy	1 (1.9)
Synchronous 2 team operating	665 (41.7)
Transanal initial dissection:	
Mucosectomy	83 (5.8)
Total intersphincteric	78 (5.5)
Partial intersphincteric	208 (14.7)
Pursestring	1027 (72.5)
Other*	21 (1.5)
Missing	177 (11.1)
Conversion	
Abdominal	69 (4.3)
Perineal	21 (1.3)
Both abdominal and perineal	12 (0.8)
Stoma	
No defunctioning stoma	177 (11.7)
Ileostomy	1282 (85.0)
Colostomy	50 (3.3)
Missing	85 (5.3)
Anastomotic technique	
Manual	512 (34.0)
Stapled	996 (66.0)
Missing	86 (5.4)
Stapled anastomoses	
Stapled configuration	
End-to-end	485 (49.6)
Side-to-end	433 (44.3)
Colonic J pouch	24 (2.5)
Ileal pouch-anal anastomosis	36 (3.6)
Missing	18 (1.8)
Manual anastomoses	
Manual configuration	
End-to-end	334 (65.2)
Side-to-end	136 (26.6)
Colo-anal J pouch	30 (5.9)
Ileal pouch-anal anastomosis	12 (2.3)
Height of anastomosis from anal verge in cm, median (range)	
Manual	2.0 (0–9.0)
Stapled	4.0 (0–11.0)
Operative time, mean ± SD (range)	
Total operative time, hours:minutes	4:12 ± 1:42 (0:30–12:13)
Perineal phase time, hours:minutes	2:03 ± 1:03 (0:14–7:47)
Intraoperative adverse events	
Technical problems during transanal phase	330 (18.0)
Incorrect dissection plane	91 (5.7)
Pelvic bleeding >100 mL	67 (4.2)
Visceral injuries during transanal phase, total	28 (1.8)
Urethral injury	12 (0.8)
Rectal tube perforation	7 (0.4)
Vaginal perforation	5 (0.3)
Hypogastric nerve divisions	2 (0.1)
Bladder perforation	2 (0.1)

Percentages for missing values use the total number of cases as the denominator (ie, 1594). Percentages for the variables are calculated out of the total number of actual results available excluding the missing values.

*Other transanal phase surgical approaches include extra-levator dissection and abdomino-perineal excision.

APE indicates abdomino-perineal excision; IPAA, ileal pouch-anal anastomosis; TME, total mesorectal excision; SILS, single incision laparoscopic surgery; SD, standard deviation.

loss > 500 mL was reported, mainly due to pelvic bleeding and splenic hemorrhage following splenic flexure mobilization. The specimen was extracted transanally in 43.9%, while abdominal extraction was utilized in the remainder either via Pfannenstiel incision (26.6%), iliac fossa/stoma site (14.8%), umbilical opening (6.7%), or the laparotomy incision (8.0%). A pelvic drain was inserted in 1134 patients (71.1%).

The commonest anastomotic technique performed was mechanical stapling in 66% with an end-to-end or side-to-end configuration in 94% of cases (Table 2). The stapler diameters used included 25 to 28 mm, 29 mm, 31 to 32 mm, and 33 mm in 14.5%, 22.3%, 17.4%, and 45.8% respectively.

Intraoperative adverse events occurred in 487 of 1594 (30.6%). Conversion to an alternative technique was required in 90 patients (5.6%). Abdominal access conversion was primarily required due to limited visualization secondary to excessive adhesions and obesity, while perineal conversions occurred after difficulty identifying the correct dissection plane leading to bleeding and/or visceral injuries. Twelve cases underwent both perineal to abdominal, and minimal access to open abdominal conversions, and were predominantly men (11/12) with a higher BMI (mean $27.1 \pm 3.9\,kg/m^2$). Table 2 outlines the incidence of technical transanal difficulties and adverse events. A total of 41 visceral injuries were recorded during both abdominal and transanal phases; 12 (0.8%) urethral injuries, 7 (0.4%) rectal tube perforation, 5 (0.3%) vaginal perforations, 5 (0.3%) ureteric injuries, 5 (0.3%) enterotomies, 3 (0.2%) bladder perforations, 2 (0.1%) hypogastric nerve divisions, 1 (0.06%) splenic injury with significant hemorrhage, and 1 (0.06%) diaphragmatic perforation during splenic flexure mobilization. Anastomosis-related technical difficulties included anastomotic defects requiring additional handsewn sutures (n = 12), complete re-do of the anastomosis due to ischemia (2) or rectal tear (1). Further intraoperative complications included injury to the mesenteric vascular arcade during attempted transanal specimen extraction, carbon dioxide embolism with hemodynamic instability, and intraoperative myocardial infarction.

Postoperative Outcomes and Anastomosis-related Morbidity

The median length of hospital stay was 8 days (range 2–94), with morbidity and mortality rates within 30 days of the primary resection of 35.4% and 0.6% respectively. Overall, 44 deaths (2.8%) have been reported over a mean follow-up period of 14 months (range 3–68). Postoperative complications within 30 days, categorized according to the Clavien–Dindo classification[20] as I/II, III, IV, and V, occurred in 354 (22.2%), 188 (11.8%), 13 (0.8%), and 9 (0.6%) patients respectively. Emergency surgical reintervention for any cause within 30 days or index admission was required in 128 (8.0%) (Supplementary Table 2, http://links.lww.com/SLA/B360: Summary of emergency operations).

Table 3 outlines the incidence of anastomosis-related morbidity, showing an overall anastomotic failure rate of 15.7%. Early AL, diagnosed within 30 days of the primary resection, occurred in 124 (7.8%) patients; 68 (61.3%) of these were managed by active therapeutic intervention without the need for a relaparotomy (Grade B). Overall 311 of 1594 (19.5%) patients required a reintervention (surgical, endoscopic, or radiological) for any cause at some point during the study period, while 135 of 311 (43.4%) of these patients required a reintervention for anastomotic failure. A total of 141 reinterventions for failure were reported during the study period. The majority, 108 of 141 (76.6%), of reinterventions for anastomotic failure involved surgery under general anesthesia, with either examination of the anastomosis with washout ± vacuum therapy, resuturing for anastomotic dehiscence, laparoscopic lavage ± defunctioning stoma or as a later reoperation with dilatation or anastomotic

TABLE 3. Anastomosis-related Morbidity

Postoperative Complications	TaTME Registry Data Results
Factor	Total: 1594 cases
Category	n (%)
Anastomotic leak:	
Early*	124 (7.8)
Delayed†	32 (2.0)
Pelvic abscess	75 (4.7)
Anastomotic fistula	12 (0.8)
Anastomotic sinus	15 (0.9)
Anastomotic stricture	58 (3.6)
Anastomotic failure‡	
Number of events diagnosed	316
Number of patients affected	250 (15.7)
Management of anastomotic failure:	
Early anastomotic leak score	
A—conservative management	23 (20.7)
B—reintervention without laparotomy	68 (61.3)
C—laparotomy required	20 (18.0)
Missing	13 (10.5)
Total number of patients requiring reinterventions due to anastomotic failure/total number of patients undergoing a reintervention at any time point	135/311 (43.4)
Total number of reinterventions for anastomotic failure at any time point	141
Type of reinterventions for anastomotic failure	
Surgical	108/141 (76.6)
Radiological	27 (19.1)
Endoscopic	6 (4.3)

*Early anastomotic leaks were diagnosed within 30-days of the primary colorectal resection.

†Delayed anastomotic leaks were diagnosed after 30-days of the primary colorectal resection.

‡Anastomotic failure is defined as the defined as the overall incidence of anastomotic-related morbidity, including early and late AL, pelvic abscess, anastomotic-related fistula, chronic sinus, and persistent anastomotic stricture following primary rectal resection.

refashioning for anastomotic stricturing. Of 250 patients diagnosed with anastomotic failure, 219 had a defunctioning stoma created at the index operation. Gut continuity was restored in 124 (56.6%). The median interval to stoma closure was 142 days (approx. $4^1/_2$ mo), range 5 to 1638 days. Twelve patients (0.8%) underwent a takedown of the anastomosis with an end stoma in the form of a Hartmann procedure for anastomotic leak (11 cases) and a completion proctectomy with end colostomy for a tight anastomotic stricture (1 case). A further six patients (0.4%) with anastomotic leaks were managed with laparoscopic washout and formation of a defunctioning stoma.

Histopathological results for the 1540 cancer cases are described in supplementary Table 3, http://links.lww.com/SLA/B360. In summary, a curative R0 resection rate was achieved in 95.7%. A positive circumferential resection margin or distal resection margin was reported in 60 (3.9%) and 10 (0.6%) cases respectively. Major defects in the TME specimen and rectal perforations were noted in 75 (4.9%) specimens.

Risk Factors for Early Anastomotic Leak

Univariate analysis identified 8 patient-related and 5 technical risk factors (P value ≤ 0.100) for early AL (Table 4). On multivariate analysis, 7 of these factors remained statistically significant. Patient-related risk factors included male sex, obesity, smoking (borderline significance), diabetes, larger tumors (>25 mm maximum diameter), and tumor height >4 cm from anorectal junction on MRI. The only significant technical risk factor was excessive intraoperative blood loss of ≥500 mL. Significantly more cases that did not have a defunctioning stoma developed an early symptomatic AL compared with those who were defunctioned (12.4% vs. 7.2%, OR 0.547, 95% CI 0.334–0.895, $P = 0.015$). Although univariate results suggested that patients who did not receive neoadjuvant therapy were at higher risk of AL and failure (Tables 4 and 5), these findings were not significant on multivariate analysis and outcomes would have been confounded by the fact that significantly more patients who had neoadjuvant treatment were defunctioned (32.8% vs 58.1%, OR 2.846, 95% CI 2.042–3.967, $P < 0.001$). Defunctioning stoma was not included in multivariate analysis as previous studies have shown that the presence of a defunctioning stoma may not prevent AL, but rather reduces the consequences should an AL occur.[21] Hence, a defunctioning stoma is proposed as a strategy to reduce the adverse effects of AL and is recommended in patients with identified risk factors.

Risk Factors for Anastomotic Failure

Fourteen potential risk factors associated with anastomotic failure were identified on univariate analysis (Table 5). Eight of these (5 patient-related and 3 technical factors) remained statistically significant on multivariate analysis including male patients, obesity, smoking, diabetes, larger tumors over 25 mm, manual anastomoses, excessive blood loss of ≥500 mL, and longer perineal phase operative time of >1.5 hours. The manual technique significantly increased the risk of late stricturing (5.9% vs. 2.7%, OR 0.448, 95% CI 0.263–0.762, $P = 0.002$). The presence of a defunctioning stoma did not appear to significantly influence the incidence of anastomotic failure in this cohort (no stoma 17.5% vs. stoma 15.6% OR 0.872, 95% CI 0.576–1.320, $P = 0.516$). Multilevel regression analysis did not demonstrate any significant clustering between hospitals for anastomotic failure rates, nor alter the significant risk factors. Figure 1 shows the scoring of patient and tumor-related risk factors and the associated percentage risk of developing anastomotic failure observed in this cohort of 1594 patients treated by a TaTME technique with a low anastomosis.

DISCUSSION

Anastomotic complications can lead to significant early and long-term morbidity, with a possible adverse impact on cancer outcomes.[2,22,23] Identifying high-risk patients and implementing appropriate reduction strategies, through preoperative patient optimization, technical considerations, and focused postoperative management with early recognition of adverse signs, are key to improving patient outcomes.

In contrast to abdominal rectal resections that usually employ a stapled distal transection, TaTME involves a transanal endoscopic full rectotomy, with an open rectal stump. A number of stapled and handsewn techniques have been reported to perform an anastomosis after TaTME.[16] Most reports have small patient numbers with little data on the morbidity associated with anastomoses following TaTME.

Results from the recently commenced randomized controlled trials comparing TaTME with laparoscopic TME may provide some robust data in the future, should sufficient numbers be enrolled.[24,25] Currently, the international TaTME registry[17] provides the largest cohort of TaTME cases performed in the wider surgical community, allowing analysis and monitoring of outcomes, and incorporating outcomes from units with different levels of surgical experience. In this study 1594 TaTME cases with an anastomosis were analyzed, with an early leak rate of 7.8%. This value is higher than the previously published rate of 5.4% in the initial 720 registry cases[18]

TABLE 4. Univariate and Multivariate Analyses of Patient-related and Technical Risk Factors for Early Anastomotic Leak

		Univariate Analysis			Multivariate Analysis		
Factor Category	**Event Rate %**	**Adjusted Odds Ratio**	**95% Confidence Interval**	***P* Value**	**Adjusted Odds Ratio**	**95% Confidence Interval**	***P* Value**
Patient-related factors							
Sex	Female 4.1	1			1		
	Male 9.5	2.475	1.529–4.006	<0.001	2.173	1.331–3.548	0.002
BMI	<30 kg/m^2 6.9	1			1		
	≥30 kg/m^2 12.4	1.901	1.238–2.918	0.003	1.589	1.012–2.494	0.044
Smoker	Nonsmoker 7.0	1			1		
	Smoker 12.2	1.831	1.172–2.861	0.007	1.576	0.991–2.506	0.055
Diabetic	Nondiabetic 6.5	1			1		
	Diabetic 18.0	3.154	2.037–4.883	<0.001	2.700	1.702–4.282	<0.001
Tumor height on MRI from ARJ	≤4 cm 6.9	1			1		
	>4 cm 9.8	1.466	1.010–2.127	0.043	0.607	0.401–0.920	0.019
Tumor size	≤25 mm 5.5	1			1		
	>25 mm 10.4	1.997	1.291–3.088	0.002	1.883	1.212–2.926	0.005
ASA	I–II 6.8	1					
	III–IV 12.2	1.917	1.275–2.881	0.002			
Neoadjuvant therapy	No 9.2	1					
	Yes 6.7	0.713	0.494–1.029	0.070			
Technical factors							
Perineal dissection	Open dissection* 4.9	1					
	Endoscopic PS† 8.9	1.896	1.127–3.190	0.014			
Anastomotic height from AV	≤3 cm 6.1	1					
	>3 cm 10.4	1.779	1.194–2.651	0.004			
Pelvic bleeding	Negligible 7.5	1					
	Noticeable‡ 13.4	1.905	0.920–3.943	0.078			
Estimated blood loss	<500 mL 6.8	1			1		
	≥500 mL 25.0	4.551	1.971–10.506	<0.001	4.334	1.900–9.888	<0.001
Specimen extraction	Transanal 6.2	1					
	Abdominal 9.5	1.601	1.073–2.389	0.020			

*Open dissection includes total and partial intersphincteric and mucosectomy dissections performed open.
†PS: pursestring suture placed endoscopically.
‡Noticeable pelvic bleeding was >100 mL with 9% of cases with pelvic bleeding having >500 mL blood loss.
ASA indicates American Society of Anesthesiologists; MRI, magnetic resonance imaging; ARJ, anorectal junction; AV, anal verge.

and could be explained by an increased complexity of cases performed transanally, wider adoption of TaTME by surgeons at the start of their learning curve, or improved recording and reporting of adverse events on the registry. Over the last year, the number of surgical centers joining the registry has almost doubled with approximately 32 cases recorded per month and 35% of centers having performed less than 5 TaTME cases. Nonetheless, the leak rate remains within an acceptable range comparable to previously reported incidences in colorectal surgery.[7–10] Similarly, the overall morbidity rate of 35.4% is within recognized rates comparable to conventional abdominal TME surgery, especially when we take into account the majority of cases selected for TaTME are the more difficult low rectal cancer cases.

Although higher leak rates have been attributed to low surgical volume,[26,27] Hyman et al[28] found that even in a group of high-volume surgeons, leak rates still ranged from 1.6 to 9.9%; despite more surgical experience and high caseload. This variation may be due to the multifactorial etiology and contributing factors that lead to AL, including both nonmodifiable and modifiable patient and tumor-related risk factors. Independent risk factors identified in previous studies include male sex, smoking, obesity, preoperative radiotherapy, emergency surgery, and tumor-related factors such as distal infraperitoneal tumors, larger tumor size, and advanced tumor stage.[13,19,29–31] Our study found similar factors to be significant for AL and overall anastomotic failure, in particular male diabetic smokers with large tumors. Sorensen et al[32] reported that smoking impairs tissue healing through nicotine-induced vasoconstriction, reduced perfusion, and carbon-monoxide-induced cellular hypoxia, leading to reduced tissue oxygen and collagen deposition. Diabetes also impacts wound healing as uncontrolled hyperglycemia leads to vascular damage, resulting in decreased blood flow and cellular accumulation of toxic glucose-derived metabolites.[33]

A recent meta-analysis by Qu et al reported 4 intraoperative factors significantly associated with increased risk of AL, including longer operative time, number of stapler firings >2, intraoperative transfusions/blood loss >100 mL, and anastomotic level of <5 cm from anal verge.[31] In TaTME, the distal rectal transection does not involve multiple stapler firings and so eliminates this potential risk factor. However, excessive blood loss and longer operative time were also found to be important factors following TaTME. Interestingly, anastomotic height appeared to be associated with AL only on univariate analysis (but not overall anastomotic failure) and a higher rate of AL occurred in anastomoses at a level of >3 cm from anal verge. Similarly, higher tumors located >4 cm from the anorectal junction on MRI were found to pose a greater risk of leakage than lower tumors, and this remained significant on multivariate analysis. Colorectal surgeons are likely to have less experience in performing a transanal pursestring on an open rectal stump at a higher distance from the anal verge prior to stapled anastomosis in their early phase of the learning curve for TaTME. The lower stapled anastomoses can

TABLE 5. Univariate and Multivariate Analyses of Patient-related and Technical Risk Factors for Overall Anastomotic Failure

Factor Category	Event Rate %	Univariate Analysis			Multivariate Analysis		
		Adjusted Odds ratio	95% Confidence Interval	*P* Value	Adjusted Odds ratio	95% Confidence Interval	*P* Value
Patient-related factors							
Sex	Female 12.1	1			1		
	Male 17.4	1.537	1.129–2.092	0.006	1.419	1.030–1.955	0.032
BMI	<30 kg/m^2 14.6	1			1		
	≥30 kg/m^2 22.6	1.698	1.221–2.362	0.002	1.484	1.049–2.102	0.026
Smoker	Nonsmoker 14.7	1			1		
	Smoker 21.7	1.617	1.142–2.288	0.006	1.506	1.054–2.153	0.025
Diabetic	Nondiabetic 14.2	1			1		
	Diabetic 27.5	2.296	1.600–3.295	<0.001	1.873	1.282–2.738	<0.001
Tumor size	≤25 mm 11.5	1			1		
	>25 mm 19.1	1.813	1.313–2.504	<0.001	1.648	1.198–2.268	0.002
ASA	I–II 13.7	1					
	III–IV 23.8	1.965	1.443–2.677	<0.001			
Ischemic heart disease, IHD	No IHD 14.7	1					
	IHD 22.1	1.650	1.162–2.343	0.005			
Neoadjuvant therapy	No 17.5	1					
	Yes 14.3	0.789	0.602–1.034	0.086			
Technical factors							
Anastomotic technique	Manual 18.9	1			1		
	Stapled 14.7	0.735	0.554–0.975	0.032	0.745	0.559–0.993	0.045
Estimated blood loss	<500 mL 13.9	1			1		
	≥500 mL 34.4	3.232	1.525–6.848	<0.001	3.020	1.431–6.376	0.004
Perineal operative time	≤1.5 h 12.1	1			1		
	>1.5 h 17.9	1.576	1.033–2.404	0.034	1.554	1.031–2.343	0.035
Intraoperative problem	No 14.6	1					
	Yes 18.1	1.287	0.968–1.710	0.082			
Pelvic bleeding	Negligible 15.3	1					
	Noticeable* 23.9	1.734	0.972–3.092	0.059			
Conversion	No 15.2	1					
	Yes 23.3	1.695	1.019–2.817	0.040			

*Noticeable pelvic bleeding was >100 mL with 9% of cases with pelvic bleeding having >500 mL blood loss.
ASA indicates American Society of Anesthesiologists.

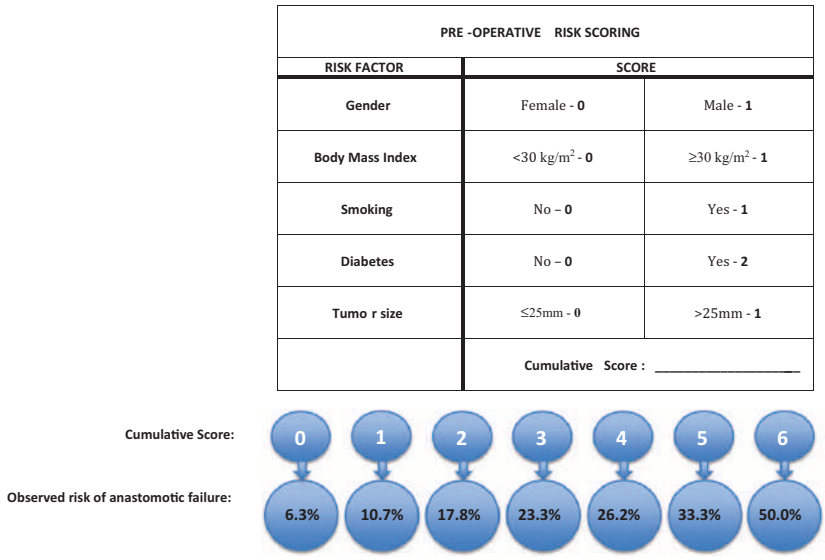

FIGURE 1. Anastomotic failure observed risk score.

also be reinforced with additional handsewn sutures that would be difficult to place more proximally, and any leakage through a lower anastomosis is more likely to discharge transanally rather than accumulating intra-abdominally with symptomatic sepsis.

The evidence regarding manual versus stapled techniques is more conflicting with no significant differences in AL rates, stricture, and mortality in colorectal anastomoses reported in a Cochrane review and recent meta-analysis.[34,35] Cong et al[36] did find significantly lower rates of AL and stricture formation following stapled compared with handsewn coloanal anastomoses after intersphincteric resection. Similarly, our results suggest that the odds of developing anastomotic failure, in particular anastomotic stricture, is 30% less likely if a stapled anastomosis is performed; although no association was noted with early AL. Depending on the degree of anastomotic stricturing, multiple interventions may be required including anastomotic dilatation, re-do anastomoses, or even conversion to a permanent stoma; all of which contribute to long-term morbidity and increased healthcare costs.

Reassuringly, 82% of TaTME patients diagnosed with an early AL were successfully managed without the need for a laparotomy. Overall 20.7% were managed conservatively and 61.3% underwent active reintervention without requiring laparotomy. Similar findings were reported by Kim et al[37] in patients with AL following minimally invasive (laparoscopic and robotic) anterior resection, with 19.7% undergoing a second open operation, while 69% and 11.3% had laparoscopic reintervention and transanal surgery respectively. The benefits of a less invasive approach, where feasible, compared with a laparotomy for AL after initial laparoscopic surgery were reported in 2 retrospective cohort studies[38,39] with shorter intensive care stay, shorter time to first diet, and earlier stoma functioning.

Reduction strategies and treatment algorithms for anastomotic failure have been developed and proposed by numerous authors and surgical societies.[7,21,40,41] The risk factors and the preoperative observed risk scoring reported in this study can aid the perioperative planning for patients undergoing TaTME. The observed risk score does however require validation which is planned on an external patient cohort in the future. Preoperative optimization with tighter glycemic control for diabetics, weight loss for the obese, and active smoking cessation programs can be initiated immediately, especially if more time is available during neoadjuvant treatment or prior to nonurgent benign resections. Operative strategies, such as the formation of a defunctioning stoma, pelvic drain placement, and use of fluorescence angiography,[42] if available to assess bowel perfusion, should be considered intraoperatively, especially if the risk score proposed here is high. Although accurate prediction of risk is impossible, appreciation of these factors may help with the discussion and decision-making with the patient as to whether an anastomosis should even be attempted, especially in the context of poor preexisting bowel function and/or poor physiological reserve to cope with anastomotic failure.

The limitations of this study include the potential for reporting bias and human error in recording registry data. Postoperative complications, in particular, may be difficult to capture, especially if patients attend a different hospital or are treated in the community. Thus, longer term outcomes are likely to be under-reported. Differences in the investigative methods to diagnose anastomosis-related pathology may further under-report the true incidence or increase heterogeneity among groups. Early leaks were also more likely to have been identified clinically and, we therefore cannot address the question of occult or subclinical leaks. However, the main intention was to determine the incidence of symptomatic leaks and to identify potential risk factors. Although the TaTME registry captures over 200 variables, certain factors that may influence anastomotic healing, such as perioperative fluid management and use of vasopressors, are not recorded. Nonetheless, at present, this registry is the largest TaTME database available and encompasses the wider surgical community performing the technique worldwide with an open and transparent collaborative.

In conclusion, anastomosis-related complications cause significant morbidity and are an ongoing challenge. New and modified anastomotic techniques have been developed to address the open stump following TaTME.[16] Analysis of the risk factors identified in this study for AL and longer term anastomotic failure aids perioperative management and decision making tailored to the patient to reduce and mitigate complications. Further research is required to determine the learning curve associated with TaTME and the optimal training pathway[43–45] to further reduce the occurrence of adverse events and to optimize the benefits of this novel access technique.

ACKNOWLEDGMENTS

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APPENDIX 1: Contributing Surgical Centers and Collaborators to the TaTME Registry Cases Reported

Country	Place of Work	Collaborators
Australia	Alfred Hospital	Stephen W. Bell Satish Warrier
	John Hunter Hospital and University of Newcastle	Peter Pockney Stephen Smith
	Peter MacCallum Cancer Centre, Melbourne, Victoria	Satish K Warrier Alexander G Heriot
	Royal Brisbane Hospital, Queensland	Andrew RL Stevenson David A Clark
Austria	St. John of God Hospital Graz	Alexander Szyszkowitz Gerald Seitinger
	Hospital Barmherzige Schwestern, Vienna	Ingrid Haunold Jakob Piehslinger
	Department of Surgery, Medical University of Vienna	Stefan Riss Anton Stift
	Franziskus Hospital, Vienna	Markus Glöckler Tobias Marcy
Belgium	Jessa Hospital Hasselt	Bert Houben Joep Knol
	University Hospital Leuven, Department of Abdominal Surgery.	André D'Hoore Albert M. Wolthuis
	AZ Groeninge, Kortrijk	Bart Van Geluwe Franky Vansteenkiste
	AZ Glorieux, Ronse	Steven Marcoen Wouter van Riel
	AZ Klina Brasschaat	Guido Jutten Pieter D'hooge
	AZ Sint-Blasius Dendermonde	Filip Vanrykel Luk Verlaeckt
	AZ Turnhout	Philippe Du Jardin Tom Hendrickx
	Ghent University Hospital	Michele Grieco Yves Van Nieuwenhove
	Department of Abdominal, Pediatric and Reconstructive Surgery, University Hospital Antwerp UZA	Niels Komen Sylvie Van den Broeck
	Ziekenhuis Netwerk Antwerpen	Frank van Sprundel Marc Janssens Yves Pirenne
Brazil	Hospital Santa Izabel, Salvador, Bahia	Carlos Ramon Silveira Mendes Meyline Andrade Lima
	Angelita & Joaquim Gama Institute, São Paulo	Guilherme Pagin São Julião Rodrigo Oliva Perez
	Progastro Institute, Campinas, São Paulo	Gustavo Sevá-Pereira
Canada	Division of General Surgery, Health Sciences North, Northern Ontario School of Medicine	Antonio Caycedo Grace Ma
	North York General Hospital and Sunnybrook Health Sciences Centre, University of Toronto, Ontario	Peter Stotland Shady Ashamalla Usmaan Hameed
	St. Paul's Hospital and University of British Columbia	Carl J. Brown Terry P. Phang
Czech Republic	Masaryk University, Faculty of Medicine and The University Hospital Brno	Martina Farkašová Tomáš Grolich Zdeněk Kala
Denmark	Roskilde Hospital	Jens Ravn Eriksen
France	Department of digestive surgery, Rouen University Hospital, Rouen	Jean-Jacques Tuech Julien Coget
Germany	Charité Universitätsmedizin Berlin, Department of Surgery, Campus Virchow-Klinikum and Campus Mitte, Berlin	Felix Aigner Matthias Biebl
	HELIOS Klinikum Wuppertal	Gabriela Möslein
	Department of General, Visceral and Transplant Surgery, University Medicine of the Johannes Gutenberg-University Mainz, Germany	Hauke Lang Werner Kneist
	Staedtisches Klinikum Karlsruhe, Klinik fuer Allgemein- und Viszeralchirurgie	Jörg Baral Theo-Julian Hoffmann
	Klinik für Allgemein-, Visceral- und Gefäßchirurgie St. Joseph Krankenhaus Berlin-Tempelhof	Lope Estévez Schwarz Reiner Kunz
Hungary	St. Borbala Hospital, Department of Surgery, Tatabánya	Balázs Bánky Miklós Lakatos
Ireland	Mater Misericordiae University Hospital	Hazar Al Furajii Ronan A Cahill

APPENDIX 1. (*Continued*)

Country	Place of Work	Collaborators
Italy	E. Agnelli Hospital, Pinerolo, Torino	Andrea Muratore Antonio La Terra
	Università Campus Bio-Medico di Roma, Rome	Gabriella Teresa Capolupo Marco Caricato
	University of Milan	Luigi Boni
	IRCCS - Ca' Granda - Policlinico Hospital	Elisa Cassinotti
	Department of Surgery, University of Rome "Tor Vergata"	Ilaria Capuano Pierpaolo Sileri
	Humanitas Gradenigo Hospital, Turin	Dario Borreca Paolo De Paolis
	Humanitas Clinical and Research Center, Colon and Rectal Surgery Unit & Humanitas University, Department of Biomedical Sciences, Rozzano, Milan	Antonino Spinelli Giulia David
	Department of Surgical Sciences, University of Torino	Alberto Arezzo Mario Morino
	"SAPIENZA" University of Rome	Emanuele Lezoche Andrea Picchetto
	Istituto Nazionale per lo Studio e la Cura dei Tumori "Fondazione Giovanni Pascale" IRCCS-Naples	Daniela Rega Paolo Delrio
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Korea	Center for Colorectal Cancer, National Cancer Center, Goyang	Dae Kyung Sohn Jae Hwan Oh
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	VU University Medical Center Amsterdam	Jaap Bonjer Jurriaan Tuynman
	IJsselland Hospital, Capelle aan den IJssel	Eelco de Graaf Pascal G. Doornebosch
	Gelderse Vallei Hospital Ede, Ede	Colin Sietses Marioes Veltcamp-Helbach
	Gelre Hospitals, Apeldoorn	Edwin S. van der Zaag Peter van Duijvendijk
New Zealand	Auckland City Hospital	Arend E H Merrie Julian L Hayes
	Waikato Hospital	Linus Wu
Norway	Department of Gastroenterologic Surgery, Akershus University Hospital, Lørenskog	Arne E. Faerden Rolf Riis
	Department of Gastrointestinal and Emergency Surgery and Department of Clinical Medicine, Haukeland University Hospital, Bergen	Frank Pfeffer Håvard M. Forsmo
Portugal	Beatriz Angelo Hospital	Susana Margarida Rodrigues Ourô Paulo Nuno Roquete Marques
Russia	Department of colorectal surgery, Blokhin Russian Cancer Research Center, Moscow	Arsen O. Rasulov Hasan E. Dzhumabaev Zaman Z. Mamedli
	Scientific Research Institute of Oncology named after N.N. Petrov, Saint-Petersburg	Aleksei Karachun Aleksei Petrov
	State Scientific Centre of coloproctology, Moscow	Evgeny Rybakov Stanislav Chernyshov
Singapore	Department of Colorectal Surgery, Singapore General Hospital	Cherylin Fu Wan Pei
Spain	Fundacio Althaia Xarxa Assistencial Universitaria de Manresa	Pablo Collera Cristina Soto
	Hospital Clinic of Barcelona	Antonio M. Lacy Borja DeLacy
	Hospital Universitario Marques de Valdecilla, Santander	Carmen Cagigas Fernández Marcos Gómez Ruiz
	Complejo Asistencial Universitario de Leon	Vicente Simó Fernández Enrique Pastor Teso
	University Hospital Fundacion Jimenez-Diaz, Madrid	Carlos Pastor Patricia Tejedor
Sri Lanka	Colombo South Teaching Hospital	Bawantha Dilshan Gamage
Sweden	Centre for Digestive Diseases at Karolinska University Hospital Solna. Department of Molecular Medicine and Surgery at Karolinska Institutet	Christian Buchli Monika Egenvall
Switzerland	Department of Visceral Surgery, University Hospital Lausanne	Dieter Hahnloser Seraina Faes
	Surgical Clinic, Limmattal Hospital, Schlieren	Alex Ochsner Urs Zingg
	Teaching Hospital Emmental, Burgdorf	Stephan Vorburger Daniel Geissmann
	Kantonsspital St. Gallen	Walter Brunner
	Department of Surgery, Cantonal Hospital Winterthur, Winterthur	Felix Grieder Michel Adamina
	Geneva University Hospitals and Medical School, Service of visceral Surgery, Department of Surgery, Geneva	Frederic Ris Nicolas Christian Buchs

APPENDIX 1. (*Continued*)

Country	Place of Work	Collaborators
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Ukraine	O.Bogomolets National Medical University, Kyiv	Miroslava Fabryko Yevgen Miroshnychenko
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	Colchester General Hospital	Greg Wynn Ralph Austin
	Ninewells Hospital & Medical School, Dundee	Ken Campbell
	St. Marks Hospital Academic Institute, North West London Hospitals NHS Trust	Danilo Miskovic Janindra Warusavitarne Pramodh Chandrasinghe
	Sandwell and West Birmingham Hospitals NHS Trust	Kathryn Gill Howard Joy
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	Medway Maritime Hospital, Kent	Henk Wegstapel Neil Kukreja
	James Paget University Hospital NHS Foundation Trust, Norfolk	Kamal Aryal Roshan Lal
	Hampshire Hospitals NHS Foundation Trust, Basingstoke	Arcot Venkatsubramaniam Steve Arnold
	North Bristol NHS Trust	Anthony Dixon Kathryn McCarthy
	University Hospitals Birmingham NHS Foundation Trust	Shazad Ashraf Simon Radley
	University Hospitals of Leicester	Baljit Singh Sanjay Chaudhri
	Wrightington, Wigan and Leigh NHS Foundation Trust	Marius T. Paraoan
	Good Hope Hospital, Heart of England NHS Foundation Trust	Stephan Korsgen
	Musgrove Park Hospital, Taunton	Paul Mackey Tom Edwards
	Queen Elizabeth University Hospital, Glasgow	Ahmed Alani Richard Molloy
	Royal Cornwall hospital	Denzil May Paul Lidder
	Royal Marsden NHS Foundation Trust and Chelsea and Westminster Hospital NHS Foundation Trust	Sarah Mills Oliver Warren
	Royal Preston Hospital, Lancashire Teaching NHS Foundation Trust	Tarek A Salem Hany
	Royal Surrey County Hospital NHS Foundation Trust	Andrea Scala Timothy A. Rockall
	Royal United Hospital Bath NHS Foundation Trust	Edward Douglas Courtney Stephen Dalton
	Yeovil District Hospital NHS Foundation Trust	Andrew S Allison Nader K Francis
	University Hospital of North Midlands	Robin Dawson Veera Garimella
	University Hospital Southampton	John Knight
	Brighton and Sussex University Hospital	William Frederick Anthony Miles Etienne Maurice Moore
	Royal Devon and Exeter NHS Foundation Trust	Stephen D. Mansfield William M. Chambers
	St James's University Hospital, Leeds	Julian Hance Sushil Maslekar
	Norfolk and Norwich University Hospital NHS Foundation Trust	Irshad Shaikh Chris Speakman
	Western Sussex NHS Foundaton Trust, Worthing Hospital	Mirza Khurrum Baig
	Worcestershire Royal Hospital	Deborah Nicol Steve Pandey
United States of Americas	Florida Hospital, Orlando	Matthew Albert Sam Atallah
	Massachusetts General Hospital & Harvard Medical School, Boston	Liliana Bordeianou David Berger
	Mount Sinai Hospital, New York	Patricia Sylla

APPENDIX 2: Definitions and References of Variable Parameters Used

Parameter	Definition
Anastomotic Leak	Classified according to the 'International Study Group of Rectal Cancer' definition[19] stating that ''anastomotic leakage should be defined as a defect of the intestinal wall at the anastomotic site (including suture and staple lines of neorectal reservoirs) leading to a communication between the intra- and extraluminal compartments.''
Anastomotic Leak Severity Grading System	Classified according to the 'International Study Group of Rectal Cancer' severity grading system [19] stating that ''Severity of anastomotic leakage should be graded according to the impact on clinical management. Grade A anastomotic leakage results in no change in patients' management, whereas grade B leakage requires active therapeutic intervention but is manageable without re-laparotomy. Grade C anastomotic leakage requires re-laparotomy''.
Anastomotic Failure	The combined rate of early and delayed anastomotic leaks, pelvic abscesses, chronic sinuses, anastomotic fistulae and strictures.
Abdominal conversion	Conversion from a laparoscopic or robotic approach to an open or transanal approach.
Perineal conversion	Conversion from a transanal approach to the mesorectal dissection to an abdominal approach; either open, laparoscopic or robotic.
Positive Distal resection margin, DRM	Presence of tumor cells within 1 mm from the excised distal end of the specimen.
Positive Circumferential resection margin, CRM	Presence of tumor cells within 1 mm from the excised nonperitonealized surface of the rectum.
Resection status	R0 – fully resected tumor R1 – microscopic presence of tumor cells at the distal or circumferential resection margins or within a lymph node <1 mm from the mesorectal fascia. R2 – Macroscopically incomplete tumor resection.
Quality of TME specimen	Using the Quirke grading system for completeness of mesorectal dissection,[*,†] each TME specimen is graded as having either an intact mesorectum, minor or major defects.

[*]Nagtegaal ID, van de Velde CJ, van der Worp E, et al. Macroscopic evaluation of rectal cancer resection specimen: clinical significance of the pathologist in quality control. *J Clin Oncol* 2002;20:1729–34.

†Quirke P, Steele R, Monson J, et al. Effect of the plane of surgery achieved on local recurrence in patients with operable rectal cancer: a prospective study using data from the MRC CR07 and NCIC-CTG CO16 randomised clinical trial. *Lancet* 2002;373:821–8.

DRM indicates distal resection margin; TME, total mesorectal excision.

ORIGINAL CONTRIBUTION

Carbon Dioxide Embolism Associated With Total Mesorectal Excision Surgery: A Report From the International Registries

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See "Editorial" on page 777.

BACKGROUND: Carbon dioxide embolus has been reported as a rare but clinically important risk associated with transanal total mesorectal excision surgery. To date, there exists limited data describing the incidence, risk factors, and management of carbon dioxide embolus in transanal total mesorectal excision.

OBJECTIVE: This study aimed to obtain data from the transanal total mesorectal excision registries to identify trends and potential risk factors for carbon dioxide embolus specific to this surgical technique.

DESIGN: Contributors to both the LOREC and OSTRiCh transanal total mesorectal excision registries were invited to report their incidence of carbon dioxide embolus. Case report forms were collected detailing the patient-specific and technical factors of each event.

SETTINGS: The study was conducted at the collaborating centers from the international transanal total mesorectal excision registries.

MAIN OUTCOME MEASURES: Characteristics and outcomes of patients with carbon dioxide embolus associated with transanal mesorectal excision were measured.

RESULTS: Twenty-five cases were reported. The incidence of carbon dioxide embolus during transanal total mesorectal excision is estimated to be ≈0.4% (25/6375 cases). A fall in end tidal carbon dioxide was noted as the initial feature in 22 cases, with 13 (52%) developing signs of hemodynamic compromise. All of the events occurred in the transanal component of dissection, with mean (range) insufflation pressures of 15 mm Hg (12–20 mm Hg). Patients were predominantly (68%) in a Trendelenburg position, between 30° and 45°. Venous bleeding was reported in 20 cases at the time of carbon dioxide embolus, with periprostatic veins documented as the most common site (40%). After carbon dioxide embolus, 84% of cases were completed after hemodynamic stabilization. Two patients required cardiopulmonary resuscitation because of cardiovascular collapse. There were no deaths.

LIMITATIONS: This is a retrospective study surveying reported outcomes by surgeons and anesthetists.

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Editorial Disclaimer: Coauthor Dr. John Monson is Coeditor for *Diseases of the Colon and Rectum.* The peer-review process for this Original Contribution was managed by a different editor and not Dr. Monson. TaTME Registory Collaborators Karim Alavi, Willem Bemelman, Justin Maykel, Alessandro Fichera, Evangelos Messaris, Ian Paquette, Rodrigo Oliva Perez, Antonino Spinelli, Scott Steele are on the DCR Editorial Board and were not involved in the peer review process.

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CONCLUSIONS: Surgeons undertaking transanal total mesorectal excision must be aware of the possibility of carbon dioxide embolus and its potential risk factors, including venous bleeding (wrong plane surgery), high insufflation pressures, and patient positioning. Prompt recognition and management can limit the clinical impact of such events. See **Video Abstract** at http://links.lww.com/DCR/A961.

KEY WORDS: Carbon dioxide embolus; Rectal surgery; Registry report; Transanal; Transanal total mesorectal excision.

Carbon dioxide embolus (CDE) is an uncommon but potentially lethal complication of minimally invasive surgery[1,2] and has been reported as a rare intraoperative occurrence during transanal total mesorectal excision (TaTME).[3] TaTME is increasingly undergoing worldwide adoption and is establishing itself as an alternative technique for total mesorectal excision (TME), as opposed to a pure abdominal approach.[4,5] While implementing any new approach, it is important to evaluate and understand potential new or significant complications to ensure patient safety and community learning. We present a report on the incidence, operative factors, and management of CDE during TaTME surgery.

PATIENTS AND METHODS

Cases on the International TaTME registries from the LOREC (tatme.medicaldata.eu) and OSTRiCh (www.ostrich-consortium.org) platforms were analyzed for incidents of CDE. The LOREC registry has data recorded from 169 medical treatment units across 42 countries, and the OSTRiCh registry has data recorded from 52 units across 4 countries. All of the registry collaborators were individually contacted to confirm their number of TaTME cases and to report any incidents of CDE. An initial e-mail was sent introducing the study, followed by a case report form for those recording incidents. Characteristics and outcomes of the 25 CDE cases were explored in more detail. Three subsequent reminders were sent to collaborators. Data were collected and analyzed using SPSS version 24 (IBM Corp, Armonk, NY). All of the categorical data are presented as number of cases and percentages, whereas continuous data are shown as either mean ± SD (range) or median with range.

RESULTS

Confirmatory responses were received from 168 international centers (76%) collaborating with the TaTME databases on the LOREC and OSTRiCh platforms covering TaTME practice in 42 countries worldwide. Seventeen of these centers from 10 different nations reported experiencing a total of 25 cases of CDE, with 1, 2, or 3 events occurring in 12, 2, and 3 centers. Based on this cohort, the incidence of CDE during TaTME surgery was estimated to be 0.4% (25/6375 cases from reporting centers).

TABLE 1. Characteristics and cases

Patient and tumor characteristics	*CO_2 emboli cases (N = 25 cases)*
Sex, n (%)	
Men	19 (76)
Women	6 (24)
Age, mean ± SD (range), y	60.3 ± 15.3 (27–83)
BMI, mean ± SD (range),kg/m^2	27.4 ± 4.0 (20.0–36.9)
Presence of relevant comorbidities, n (%)	
Hypertension	9 (36.0)
Ischemic heart disease	4 (16.0)
Atrial fibrillation	1 (4.0)
Lung disease (COPD/asthma)	4 (16.0)
Previous abdominal or pelvic surgery, n (%)	6 (24.0)
Cancer cases, n (%)	20 (80.0)
Tumor height from anorectal junction on MRI, median (range), cm	4.0 (0–12)
Preoperative MRI staging, n (%)	
≥mrT3	12 (60.0)
mrN+	10 (50.0)
Received neoadjuvant therapy, n (%)	15 (60.0)
Type of neoadjuvant therapy, n (%)	
Chemoradiotherapy	13 (86.7)
Short-course chemoradiotherapy	1 (6.7)
Chemotherapy alone	1 (6.7)

Patient and Tumor Characteristics

Table 1 outlines the main patient and tumor characteristics for the TaTME procedures complicated by CDE, between June 2015 and May 2018 inclusive. CDE affected 19 male (76%) and 6 female (24%) patients. The indication for surgery was cancer or benign disease in 20 (80%) and 5 (20%) cases.

Operative and Anesthetic Characteristics

All of the CDEs occurred in the presence of transanal insufflation, either alone (10 cases, 40%) or during synchronous abdominal insufflation (15 cases, 60%). Additional operative details are outlined in Table 2. Two of the low anterior resections for cancer involved intersphincteric dissections, and 1 case included a simultaneous prostatectomy. Sacral bone resection was required in 1 abdominoperineal excision because of T4 disease with bony invasion. The case described as "mesorectal excision" in Table 2 involved a patient with ulcerative colitis who previously underwent total colectomy with subsequent close rectal completion proctectomy. An incidental rectal adenocarcinoma (T1 Sm2) was found in the proctectomy specimen, and the patient was kept under surveillance. Three years later a focus of mesorectal recurrence was identified on imaging and a decision made to proceed to transanal excision of the mesorectum.

A flexible transanal platform was used in all cases, and in 24 of 25 cases an AirSeal System (CONMED, Utica, NY) was used; the other case used another high-pressure insuffla-

TABLE 2. Operative details

Operative characteristics	*CO_2 emboli cases (N = 25 cases)*
Indication, n (%)	
Cancer	20 (80)
Benign	5 (20)
Cancer cases, n (%)	
Anterior resections ± stoma	16 (80)
Abdominoperineal resections	3 (15)
Mesorectal excision	1 (5)
Benign cases, n (%)	
Restorative proctocolectomy + IPAA for UC	1 (20)
Total proctocolectomy for UC	1 (20)
Completion proctectomy + IPAA for UC	1 (20)
TAMIS redo of anastomosis for chronic sinus after anastomotic leak	1 (20)
Redo anterior resection, rectovaginal fistula repair with loop ileostomy	1 (20)
Transanal platform, n (%)	
GelPOINT path	20 (80)
GelPOINT mini	5 (20)
Transanal energy source, n (%)	
Monopolar cautery	20 (80)
Bipolar cautery	1 (4)
Energy device (Harmonic/Ligasure)	4 (16)
Transanal insufflation system, n (%)	
Airseal	24 (96)
High pressure ventilation	1 (4)
At time of CO_2 embolus event	
Operating teams, n (%)	
Transanal operating only	10 (40)
Synchronous abdominal and transanal operating	15 (60)
Insufflation pressures, median (range), mm Hg	
Abdominal	8.0 (0–16)
Transanal	15.0 (12–20)
Bleeding vessel at time of CO_2 embolus, n (%)	
Peri/prostatic vein	10 (40)
Para/vaginal vein	3 (12)
Lateral pelvic vein	5 (20)
Posterior pelvic vein	2 (8)
Inferior mesenteric artery	1 (4)
No bleeding identified	4 (16)

UC = ulcerative colitis; TAMIS = transanal minimally invasive surgery.

tion system. Visible bleeding was present in 21 cases (84%) at the time of the event; 20 were identified transanally with predominantly venous bleeding, whereas 1 case reported abdominal bleeding from the inferior mesenteric artery.

All of the patients had general anesthesia with either total intravenous anesthesia (n = 11 (45%)) or volatile anesthesia (n = 14 (55%)) maintenance. Analgesia was provided predominantly with short-acting opiates (fentanyl and remifentanil), whereas relatively few patients had adjunctive neuraxial blockade (n = 6 (24%)). Most patients were paralyzed during surgery, using either rocuronium (n = 17 (68%)) or atracurium (n = 3 (12%)). A relatively large proportion of patients had invasive monitoring placed at induction, perhaps reflecting the potential for prolonged surgery in this cohort (64% (n = 16) had invasive blood pressure monitoring placed, and 20% (n = 5) had both central lines and arterial lines). In contrast, esophageal echocardiography and precordial Doppler sonography were used in only 1 case each. The mean volume of intravenous fluid administered before CDE was 898 mL (range, 200–2500 mL).

Intraoperative Detection and Management of CDE

At the time of CDE, most patients had legs elevated and were supine in the Trendelenburg position between 30° and 45° (n = 17 (68%)) or between 5° and 25°(n = 6 (24%)). One patient was positioned flat at the time of CDE. The earliest clinical sign identified in most cases was a reduction in the end tidal CO_2 ($ETCO_2$; n = 22 (88%)). Although the degree of $ETCO_2$ reduction considered significant was not defined a priori within data collection, subsequent correspondence has suggested that a reduction by >30% was typical. Arterial blood gases analysis after CDE typically showed a respiratory acidosis and hypoxia, defined as an oxygen saturation <92%. After CDE, urgent intraoperative transthoracic echocardiography was performed in 8 cases (32%). In all of these cases, echocardiography identified gas bubbles in the heart. After echocardiography, 3 patients had emergency insertion of a central venous catheter with attempted aspiration of gas bubbles before continuation of the operation. In 1 instance, bilateral chest drains were inserted erroneously for suspected pneumothoraxes because of the acute desaturation of the patient. In the majority of cases (n = 21 (84%)), the operation was continued after re-establishing hemodynamic stability. A conversion to open surgery occurred in 7 cases (28%), and 13 cases (52%) switched from a transanal approach to a top-down laparoscopic approach. Four cases were terminated and continued at a later time. The first was because of cardiac arrest requiring cardiopulmonary resuscitation (CPR) and gas in all 4 of the cardiac chambers. This patient had a temporary closure with surgery completed the following day. Another case was terminated because of cardiovascular collapse requiring transient CPR shortly after bleeding after completion of the rectotomy. The completion proctectomy was rescheduled for a later date. A third patient underwent laparotomy 8 hours later after 2 hours of hyperbaric chamber therapy in another hospital. The final patient required management of ventricular tachycardia and fibrillation on the intensive care unit with completion of the operation the following day. A planned restorative procedure was changed to a Hartmann procedure in 2 patients.

Postoperative Recovery

Unplanned admission to the intensive care unit or high-dependency unit was necessary in 15 patients (60%) after surgery. According to Clavien–Dindo grading,[6] 12 patients (48%) experienced a postoperative complication, including grade I in 1 case (surgical site infection), II in 7 cases (wound infection, prolonged ileus, pneumonia, urinary retention, urinary tract infection), IIIa in 6 cases (pelvic col-

lections requiring image-guided drainage, vesicourethral anastomotic leak after concomitant prostatectomy), IIIb in 2 cases (pelvic collection drained through rectal stump, suturing of partial anastomotic dehiscence), and IV in 2 cases (renal failure requiring intensive care support, saddle pulmonary embolus requiring heparin infusion and catheter-directed thrombolysis). No deaths occurred during the index admission. The mean length of stay was 12.0 ± 8.8 days ((mean ± SD) median = 9 d; range, 1–36 d).

DISCUSSION

These data provide a descriptor of CDEs during TaTME with the aim of determining a degree of commonality between cases and increasing awareness of this problem. Based on this cohort, the incidence of clinically apparent CDE is estimated at 0.4% in TaTME surgery.

The incidence of CDE has not been reported specifically in relation to other techniques for TME. However, it is clear from the literature that CDE is not a phenomenon specific to TaTME, with evidence available across a broad variety of laparoscopic and robotic cases.[7–12] Because of different detection methods and classification according to clinical significance, there are mixed reports on the prevalence of CDE during laparoscopy. One meta-analysis from 1997 of 489,335 laparoscopic cases between 1974 and 1997 reported an incidence of 0.001%.[13] However, this figure was based on the number of fatalities attributed to CDE. As monitoring equipment has evolved, the detection rates for venous gas embolism (VGE) have increased. Indeed, when highly sensitive equipment is used, clinically insignificant CDE appears almost ubiquitous to some laparoscopic cases. Studies specifically using the most sensitive method, transesophageal echocardiography (TEE), have documented rates ranging from 6% to 100% across a breadth of minimally invasive procedures.[14–17] The report of a 100% incidence (40/40 patients) comes from 1 case series of laparoscopic hysterectomy.[18] Interestingly, in this nonrandomized study VGE was found universally in the laparoscopic group, but there was also an incidence of 15% even in the open group (all grade I VGEs; see Table 3.[19]). It should, however, be noted that in open surgery gas emboli are attributed to air and not just CO_2 alone and may consequently take longer to absorb. One study comparing robotic versus open prostatectomy found the incidence to be 80% in the open arm compared with 38% in the robotic arm,[9] yet no events reached clinical significance hemodynamically. In the present report we describe the incidence of CDE in TaTME surgery based on clinical signs and symptoms, and similar large case series reporting on the same outcomes are rare. Although clinically relevant CDEs in TME surgery have not been reported, 1 study in gynecological laparoscopy has estimated an incidence of 0.59% (7/1194) based on clinical findings alone.[20]

TABLE 3. Stages of venous gas embolism

Stage	*Findings on TOE*
0	No emboli
1	Several gas bubbles in RA, RV, and RVOT
2	Gas emboli less than half the diameter of RA, RV, and RVOT
3	Gas emboli more than half the diameter of RA, RV, and RVOT
4	Gas emboli completely filling the diameter of RA, RV, and RVOT

TOE = transesophageal echocardiogram; RA = right atrium; RV = right ventricle; RVOT = right ventricular outflow tract.

Various adjuncts exist to aid in the detection of CDE. The gold standard is TEE, which has the ability to detect gas bubbles 5 to 10 μm in size at a concentration of 0.02 mL/kg.[21] Although TEE is the most sensitive detection method, it should be appreciated that such trivial amounts of gas may never reach clinical significance. Monitoring for coronary embolism (CE) using TEE may be feasible in most centers. However, TEE is also invasive, expensive, and, when used in a diagnostic capacity, requires an expert operator, meaning that its routine use has not been adopted for the majority of surgical procedures.[22] Although none of the cases in our report used TEE for routine monitoring, 32% of cases described the use of transthoracic echocardiography to confirm multiple gas emboli during a suspected event. This suggests that its routine use may offer a means of early detection and could therefore potentially prevent evolution to a clinical relevant CDE with cardiovascular compromise. This might be particularly relevant in comorbid patients with cardiorespiratory disease who are probably more susceptible to a low-volume embolus. Other detection methods include pulmonary artery catheterization, precordial Doppler sonography, end-tidal nitrogen monitoring, and $ETCO_2$ monitoring. Although the latter is not entirely specific for CDE, $ETCO_2$ is perhaps the most readily available and accessible method for detection, with early changes of as little as 2 mm Hg indicative of an event.[19] From this report we find that the detection and management of CDE in TaTME reflect the existing literature. A reduction in $ETCO_2$ was most frequently recorded as the first sign of embolism, typically a reduction by >30% and with approximately half of patients showing some form of cardiovascular compromise.

It is evident that the causality of CDE in TaTME is multifactorial and presently unclear, yet it shares many risk factors with other techniques for pelvic dissection. Operating in close proximity to Batson's venous plexus around the prostate with its network of valveless veins has drawn particular attention.[23] In fact, 1 previous study examining CDE during open and robotic prostatectomy reported that the majority of incidents occurred during dissection of the dorsal prostatic venous plexus.[9] In this TaTME report we find that, in these cases of hemodynamically significant

CDE, the majority of events (84%) occurred when venous bleeding was evident during the transanal component of dissection, with bleeding from periprostatic veins (40%) being the most common site. The transanal component of dissection around prostatic veins should therefore warrant extra caution in any TaTME case. We note also that 25% of CEs occurred in female patients. In women the para/vaginal veins exist as an anatomic equivalent, and bleeding from this venous plexus may also factor in the development of CE. The authors speculate that, in most cases, a tangential venous injury occurs, which means the injury site is less likely to fully collapse by the insufflation pressure, because it is splinted open by surrounding tissue. This will allow for ongoing CO_2 entrainment, although there might not be any visible bleeding.

Another contributory factor perhaps more unique to TaTME is the small operative space, particularly early on in the dissection, in combination with insufflation. Interestingly, although surgical planes are different, there are no reports of CDE during other minimally invasive transanal techniques, such as local excision by transanal endoscopic microsurgery or transanal minimal invasive surgery, possibly because of the potential for gas to decompress into the proximal colon.

Patient positioning is also an important factor. As with other laparoscopic or robotic approaches to pelvic dissection, the patient is often placed in the Trendelenburg position with the operative field above the right heart. The resultant pressure gradient can promote CO_2 entrainment into the venous system. However, some studies evaluating robotic prostatectomy have suggested that the use of steep Trendelenburg, for example >30°, may in fact be protective because of an increase in right heart pressure preventing venous gas entrainment.[9] In the present series, 68% of cases document the patient being placed in the head-down position at 30° to 45°. Recent evidence suggests that the degree of elevation is directly related to the rate of gas embolism.[24] However, there are no documented reports of CDE in other approaches to TME surgery, such as top-down laparoscopy or robotic techniques, while using similar patient positions.

Pneumatic CO_2 insufflation has been well analyzed in relation to TaTME surgery.[25] When there is an evident venotomy during surgical dissection, the surgeon and anesthetist should be vigilant for signs of CDE.[26] Because insufflation pressures usually exceed central venous pressure, bleeding may not always be initially apparent. It should be noted that CO_2 remains the safest gas for insufflation because of its rapid rate of absorption, and therefore unless gas reaches the coronary or cerebral arteries, small volumes are usually insignificant. However, undetected or uncontrolled venous injury, with ongoing insufflation, can cause significant gas accumulation in the circulation. All cases in this series used insufflation management units in place of standard insufflation as is now common and recommended in TaTME surgery. These units provide continuous pressure sensing, which maintains a stable pneumopelvis to

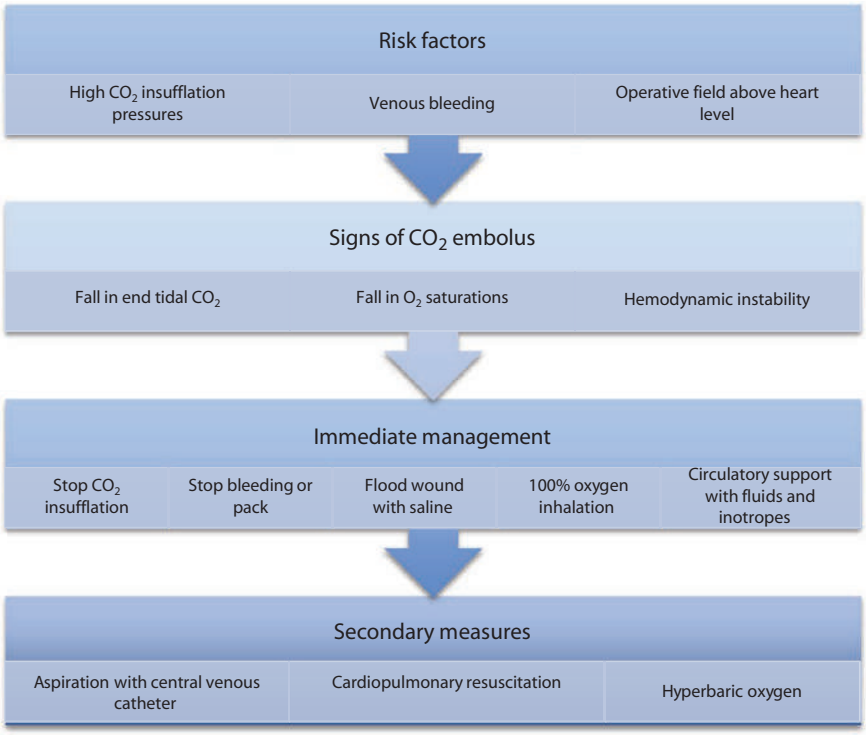

FIGURE. Risk factors, signs and immediate management of CO_2 embolism during transanal total mesorectal excision.

facilitate a consistent view while preventing cyclic billowing along with continuous smoke extraction during transanal dissection.[27] From the 25 cases described, the authors are unaware of any reports submitted to the US Food and Drug Administration Manufacturer and User Facility Device Experience. In 24 of 25 cases of CE, the Airseal device was used, which could reflect the widespread adoption of these particular devices in cases added to the TaTME registries. However, because of their regularity of use, it cannot be ignored that these devices may play a causative role in the frequency of CDE seen with TaTME dissection. Particular caution should be applied during periods of prolonged suction, especially during bleeding, because of the fact that these units will immediately compensate for pressure loss by initiating a high flow of CO_2 (≈40 L/min)[25] to prevent the collapse of the operative field. Although is widely accepted that CO_2 insufflation significantly contributes to the formation of CDE, we cannot attribute a specific level of risk with these devices without a controlled scientific evaluation with an active comparison group. The fact that a higher proportion of events occurred during synchronous transanal and transabdominal mesorectal dissection may suggest that the cumulative effect of smaller emboli through differing sites of gas entrainment may be a contributory factor. Likewise, higher insufflation pressures may need to be used during synchronous dissection to maintain an optimal view in the transanal field, although reported median transanal and transabdominal pressures at the time of embolus were 15 and 8 mm Hg.

Expert guidelines from the American Heart Association exist on the immediate management of patients presenting with CDE with cardiovascular collapse.[28] The principles of managing CDE involve the prevention of further gas entrainment into the circulation, removal of existing CO_2, and supportive measures for the patients' cardiovascular and respiratory system, including the use of inotropes (Figure). CO_2 insufflation should be stopped and/or the operative field flooded with saline to prevent additional gas entry.[26] The bleeding site should subsequently be controlled by monopolar/bipolar energy or local compression with a small gauze. Repositioning the patient may prevent additional CO_2 entry. Uncertainties surrounding patient positioning in the literature are perhaps reflected in this TaTME series, with half of the cases opting to level the operating table and a fifth opting to increase the Trendelenburg angulation. There are mixed results from reported attempts to aspirate gas from the right heart through a suitably placed multilumen catheter. Animal studies report success rates ranging from 6% to 80%,[29–31] and it is apparent that there was some benefit in the present case series (Table 4). If necessary, CPR and chest compressions should be instigated in situations of cardiovascular collapse and to help disperse gas bubbles trapped in the right heart and pulmonary arterial system.

After immediate management of CDE, the majority of procedures were continued without a significant impact on the operative course. Clearly this decision should be guided by the patient's hemodynamic condition, but in 84% of reports, surgery soon recommenced. None of the events recorded resulted in death and direct association with the reported grade III to IV postoperative morbidity is unlikely. However, it is disturbing that in 2 cases a restorative procedure was changed to a Hartmann procedure. The severity and potential lethality of this complication are further underlined by the requirement of on-table CPR in 2 patients. The insertion of chest tubes in 1 case also highlights a need for awareness of CDE among surgeons and anesthetists to reduce the occurrence of potentially avoidable invasive interventions and to ensure the correct management of these incidents.

Prevention of a clinically significant CDE deserves probably even more attention. The authors would recommend surgeons to reduce the transanal pressure setting to a very low level or even to stop insufflating if there is any

TABLE 4. Surgical and anesthetic management of intraoperative CO_2 emboli

Intraoperative management of CO_2 emboli	*CO_2 emboli cases (N = 25 cases)*
Surgical management, n (%)	
Immediate management	
Abdominal insufflation switched off	10 (66.7)
Transanal insufflation switched off	22 (88.0)
Operating table flattened	14 (56.0)
Operating table placed more in Trendelenburg position	6 (24.0)
Operating table placed in anti-Trendelenburg position	1 (4.0)
Pelvis packed to control bleeding	13 (52.0)
Bleeding stopped endoscopically transanally	8 (32.0)
Bleeding stopped transanally open	6 (24.0)
Operative progress	
Operation terminated, n (%)	4 (16.0)
Operation temporarily paused, n (%)	17 (68.0)
Operative delay (mean ± SD), min	31 ± 17.9
Minimal delay to the operation, n (%)	4 (16.0)
Conversion, n (%)	
Abdominal (MIS to open)	7 (28.0)
Transanal (stopped transanal procedure)	13 (52.0)
Anesthetic management	
Initial clinical signs identified, n (%)	
Reduction in tidal CO_2 volume	22 (88.0)
Hypotension	14 (56.0)
Reduced oxygen saturation	12 (48.0)
Immediate management, n (%)	
High flow oxygen	25 (100.0)
Administration of vasoactive drugs	22 (88.0)
Central line insertion and aspiration	3 (12.0)
Echocardiogram	8 (32.0)
CPR	2 (8.0)
Bilateral chest drains for suspected pneumothoraxes	1 (4.0)
Left needle thoracostomy for suspected pneumothorax	1 (4.0)
Hyperbaric chamber therapy	1 (4.0)

MIS = minimally invasive surgery; CPR = cardiopulmonary resuscitation.

concern of a venous injury. A small tangential vein injury, which might not cause bleeding during a transanal pressure of 12 to 15 mm Hg, could become apparent and then allow for immediate control before causing a clinical relevant CDE. Clear communication between the transanal surgical team and the anaesthetic team is essential, particularly in challenging cases with a higher risk of injury to venous vessels. Any change in $ETCO_2$ or sudden hemodynamic alteration should be immediately communicated to allow appropriate action to be taken. Of course, an understanding of bottom-up anatomy and avoidance of wrong plane surgery are fundamental to avoid CDE.

As a retrospective study relying on the completion of a questionnaire, this study does have certain limitations and finds itself susceptible to ascertainment, reporting, and recall bias. Data recording in the LOREC and OSTRiCh TaTME registries is voluntary and relies on the honest and regular contribution by its collaborators. The authors did e-mail all collaborators with repeated reminders to verify and confirm data entered and to obtain as much information as possible about the CDE cases. Furthermore, the true incidence of CDE in TaTME may also be underestimated, because some cases may not have been recognized as being caused by a CDE and may have been labeled with a different diagnosis. Still, the case series presented here is the largest to date and provides valuable data for surgeons and anesthetic teams involved in TaTME procedures.

CONCLUSION

To date this article provides the first international case series review of CDE in TaTME surgery. Factors that surgeons should pay particular attention to are venous injuries, steep head-down positioning, and high insufflation pressures. Additional data will help to further evaluate the incidence of CDE, and we encourage all incidents of suspected or confirmed CDE to be recorded on the TaTME registries.

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Predictive Factors and Risk Model for Positive Circunferential Resection Martgin after Transanal Total Mesorectal excision in 2653 Patients with Rectal Cancer

Predictive Factors and Risk Model for Positive Circumferential Resection Margin Rate After Transanal Total Mesorectal Excision in 2653 Patients With Rectal Cancer

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Objective: The aim of this study was to determine the incidence of, and preoperative risk factors for, positive circumferential resection margin (CRM) after transanal total mesorectal excision (TaTME).

Background: TaTME has the potential to further reduce the rate of positive CRM for patients with low rectal cancer, thereby improving oncological outcome.

Methods: A prospective registry-based study including all cases recorded on the international TaTME registry between July 2014 and January 2018 was performed. Endpoints were the incidence of, and predictive factors for, positive CRM. Univariate and multivariate logistic regressions were performed, and factors for positive CRM were then assessed by formulating a predictive model.

Results: In total, 2653 patients undergoing TaTME for rectal cancer were included. The incidence of positive CRM was 107 (4.0%). In multivariate logistic regression analysis, a positive CRM after TaTME was significantly associated with tumors located up to 1 cm from the anorectal junction, anterior tumors, cT4 tumors, extra-mural venous invasion (EMVI), and threatened or involved CRM on baseline MRI (odds ratios 2.09, 1.66, 1.93, 1.94, and 1.72, respectively). The predictive model showed adequate discrimination (area under the receiver-operating characteristic curve >0.70), and predicted a 28% risk of positive CRM if all risk factors were present.

Conclusion: Five preoperative tumor-related characteristics had an adverse effect on CRM involvement after TaTME. The predicted risk of positive CRM after TaTME for a specific patient can be calculated preoperatively with the proposed model and may help guide patient selection for optimal treatment and enhance a tailored treatment approach to further optimize oncological outcomes.

Keywords: circumferential resection margin, rectal cancer, registry, risk factors, transanal total mesorectal excision

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Total mesorectal excision (TME), as first described by Heald in 1982, established what is now considered optimal surgical treatment for patients with resectable rectal cancer.[1] Subsequently the treatment of rectal cancer has changed, with use of neoadjuvant therapy (NAT) in patients with advanced cancers, and a move toward minimal access techniques in selected cases.[2] More recently, transanal total mesorectal excision (TaTME) has been developed aiming to increase the quality of surgical resection and improve oncological outcomes, particularly in patients with low rectal cancers.

One of the fundamental principles of TME, and indeed all rectal cancer surgery, is to remove the tumor with a clear circumferential resection margin (CRM). Quirke et al have repeatedly shown that a positive CRM is associated with a significant increase in both local and systemic recurrence.[3,4] Despite some reported benefits from laparoscopic rectal resection, CRM positivity rates have not diminished over time.[5–8] Several tumor-related factors (anterior location, cT4-status) and patient-related factors (male sex, obesity),[9–13] are known to increase the technical difficulty in conventioneal laparoscopic TME, and therefore associated with a positive CRM.[14–17] However, there is little information on predictive factors for positive CRM after TaTME. Predictive factors might differ from the well-known risk factors after conventional laparoscopic resection, considering the different approach from below.

The present study aimed to determine the incidence of positive CRM after TaTME surgery for rectal cancer, for patients recorded on an international TaTME registry. Moreover, formulating a predictive model, preoperative predictive factors for positive CRM will be studied.

METHODS

Patient Selection

This was an analysis of prospective registry-based data. The study population comprised patients recorded on the international TaTME registry between July 2014 and January 2018.[18] Exclusion criteria were benign indications for TaTME, previous local excision, and cases in which CRM status was not known. The registry is a secure online voluntary database where surgeons worldwide are invited to record their TaTME cases, with an extensive collaboration among 172 centers worldwide.[19] Ethical approval for the registry was granted by the UK Health Research Authority (REC reference 15/LO/0499, IRAS project ID 156930). Before data analysis, registered surgeons were invited via email to update their patients'

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records, with multiple reminders to minimize missing data. Surgeons were individually contacted to clarify unexpected or possibly erroneously entered results.

Data Extraction and Outcome Parameters

The registry is designed to prospectively collect data on patient demographics, tumor staging and neoadjuvant treatment (NAT), operative details, postoperative clinical and histological outcomes, readmission details, late morbidity, and long-term oncologic follow-up. The main endpoints of this study were the incidence of, and predictive factors for, positive CRM, defined as the presence of tumor or a malignant lymph node 1 mm, or less, from the CRM. The TME specimen quality was categorized using the principles described by Nagtegaal et al.[20] The MRI response to NAT was scored by the tumor regression grade (TRG) classification, which was subgrouped into ''good response'' (mrTRG 1 and 2) and ''bad response'' (mrTRG 3, 4 and 5) as defined in the TRIGGER trial.[21] Patients treated with short-course radiotherapy and immediate surgery were included in the ''no-neoadjuvant group'' for the analysis of mrTRG, as this is not associated with significant tumor downstaging.

Statistical Analysis

Statistical analyses were performed in the Statistical Package for Social Sciences (SPSS) of IBM Statistics, version 24. Missing data for variables included in the model were imputed using single imputation with predictive mean. For the other variables presented that were not included in the predictive model, missing data did not exceed 15%. Percentages shown represent actual data available, excluding missing values.

Categorical variables were defined as absolute numbers of cases and percentages. Continuous data were reported as mean with standard deviation (SD). Continuous variables such as BMI and tumor height from anorectal junction (ARJ) were categorized into clinically relevant subgroups. For intergroup variation, the chi-square test was used, whereas the Mann–Whitney *U* test was used for continuous variables. Variables to be included in the multivariate analysis were chosen based on a priori known risk factors for positive CRM from the literature. Variables reaching a $P < 0.05$ using backward step selection in the multivariate regression analysis were deemed significant and included as a predictive factor for positive CRM in the predictive model. The coefficients derived from the multivariate analysis were multiplied by 10 and used as weights in the nomogram for predicting the risk of positive CRM after TaTME for rectal cancer.

Model Validation

An interval validation was performed by drawing a random sample of 150 patients from the original study population. The so-called ''bootstrap'' technique is thought to be the optimum technique of internal validation.[22] Calibration, or goodness-of-fit, refers to the ability of the model to assign the correct probabilities of outcome to individual patients. This was checked by plotting the observed number of positive CRM to the expected number of positive CRM. Discrimination refers to the ability of the model to assign higher probabilities of positive CRM to patients who actually have a positive CRM compared with patients who do not. This was tested using the area under the receiver-operating characteristic (ROC) curve with 95% confidence intervals (CIs). The performance of the prediction model was analyzed using RStudio (version 1.1.453).

RESULTS

Patient Characteristics and Pathologic Outcomes

All cases recorded on the international TaTME registry between July 2014 and January 2018 were reviewed (n = 3240).

TABLE 1. Patient and Tumor Characteristics and Pathological Outcome

Factor	TaTME Registry Data Results Total = 2653
Patient characteristics	
Mean age, yrs (SD)	64.4 (11.7)
Male sex	1827 (68.9%)
Mean BMI, kg/m^2 (SD)	26.3 (4.5)
BMI >30 kg/m^2	507 (19.1%)
ASA classification	
I	597 (22.5%)
II	1418 (53.4%)
III	507 (19.1%)
IV	62 (2.3%)
Previous pelvic therapies	
Hysterectomy	72 (2.7%)
Prostatectomy	66 (2.5%)
Radiotherapy	33 (1.2%)
Tumor characteristics	
Mean distance from ARJ, c, (SD)	3.8 (2.6)
Within 1 cm	523 (19.7%)
Anterior tumor	1181 (44.5%)
Clinical T-stage	
cT0	16 (0.7%)
cT1	78 (3.3%)
cT2	615 (25.6%)
cT3	1534 (63.9%)
cT4	157 (6.5%)
Clinical N-stage	
cN0	1187 (44.7%)
cN1	1015 (38.3%)
cN2	451 (17.0%)
EMVI positive on baseline MRI	895 (33.7%)
Pretreatment threatened/involved CRM	674 (25.4%)
Neoadjuvant therapy	1569 (59.1%)
Short course radiotherapy immediate surgery	150/1569 (9.6%)
Long course radiotherapy delayed surgery	175/1569 (11.2%)
Long course chemoradiotherapy	1027/1569 (65.5%)
Chemotherapy alone	180/1569 (11.5%)
Contact radiotherapy	2/1569 (0.1%)
Unknown/other	35/1569 (2.2%)
TRG response post downsizing therapy*	
''Good response''	612/1419 (43%)
''Bad response''	810/1419 (57%)
Sphincter saving surgery	2442 (92.0%)
Pathological outcome	
T-stage	
pT0	293 (11.0%)
pT1	298 (11.2%)
pT2	834 (31.4%)
pT3	1126 (42.4%)
pT4	66 (2.5%)
N-stage	
pN0	1865 (70.3%)
pN1	532 (20.1%)
pN2	256 (9.6%)
Mean number of lymph node harvested (SD)	17.7 (10.3)
Mean tumor size, mm (SD)	26.1 (19.3)
Size >20 mm	1745 (65.8%)
Size >30 mm	1159 (43.7%)
CRM involvement	107 (4.0%)
DRM involvement	26 (1.0%)
TME specimen grade	
Complete	2145 (80.9%)
Near-complete	274 (10.3%)
Incomplete	89 (3.4%)
Rectal perforation	47 (1.8%)
Composite poor pathological outcome	224 (8.4%)

ARJ indicates anorectal junction junction; ASA, American Society of Anaesthesiologists-Classification; bad response, mrTRG 3, 4 and 5; BMI, body mass index; cN-stage, clinical nodal stage; CRM, circumferential resection margin, defined as involved if the distance of tumor or malignant lymph node to the mesorectal fascia was 1 mm or less; CT, chemotherapy; cT-stage, clinical tumor stage; DRM, distal resection margin; Composite poor pathology, CRM+ and/or DRM+ and/or incomplete TME specimen and/or perforations; EMVI: extramural venous invasion; good response, mrTRG 1 and 2; IQR, interquartile range; LCCRT, long course chemoradiotherapy; LCRT, long course radiotherapy; MRI, magnetic resonance imaging; pN-stage, pathological nodal stage; pT-stage, pathological tumor stage; R1, tumor or malignant node 1 mm or less from the resection margin; SCRT, short course radiotherapy (including contact radiotherapy and short course radiotherapy with delayed surgery); TME, total mesorectal excision; TRG, tumor regression grading on MRI.

*Downsizing therapy was considered as all neo-adjuvant treatment, with exclusion of patients receiving short course radiotherapy and immediate surgery (1569–150 = 1419).

TABLE 2. Multivariable Analysis of Preoperative Risk Factors for CRM Positivity in All Patients (n = 2653)

		Multivariate Analysis		
Factor	**Event Rate (%)**	**Odds Ratio**	**95% CI**	***P***
Up to 1 cm from ARJ				
Yes	38/523 (7.3%)	2.09	1.368–3.194	0.001
No	69/2130 (3.2%)	Ref.		
Anterior tumor				
Yes	62/1181 (5.2%)	1.66	1.118–2.485	0.012
No	45/1472 (3.1%)	Ref.		
cT4 tumor				
Yes	19/157 (12.1%)	1.93	1.074–3.479	0.028
No	88/2496 (3.5%)	Ref.		
EMVI on MRI				
Yes	56/895 (6.3%)	1.94	1.297–2.930	0.001
No	51/1758 (2.9%)	Ref.		
Threatened CRM on baseline MRI				
Yes	49/674 (7.3%)	1.72	1.116–2.679	0.014
No	58/1979 (2.9%)	Ref.		
Sphincter-saving surgery				
Yes	90/2442 (3.7%)	Ref.		
No	17/211 (8.1%)	1.75	0.998–3.009	0.051

ARJ indicates anorectal junction; CRM, circumferential resection margin; EMVI, extramural venous invasion; MRI, magnetic resonance imaging.

A total of 2653 TaTME cases met the eligibility criteria and were included in this analysis.

Table 1 presents patient and tumor characteristics and pathological outcome. Of the included patients, 1827 (68.9%) were male and 507 (19.1%) had a BMI of $>30\,kg/m^2$. Tumor height was within 1 cm from the ARJ in 523 (19.7%) and anteriorly located in 1181 (44.5%). Preoperative staging was reported as cT1 in 78 (3.3%), cT2 in 615 (25.6%), cT3 in 1534 (63.9%), and cT4 in 157 (6.5%). Overall, extramural venous invasion on MRI (mrEMVI), was reported in 895 (33.7%) of the cases. Threatened CRM on baseline MRI was reported in 674 (25.4%). Neoadjuvant treatment (NAT) was given in 1569 (59.1%). Patients receiving NAT to induce tumor downsizing (this excludes short-course radiotherapy with immediate surgery) obtained a ''good response'' (mrTRG 1 or2) in 612 (43%) and a ''bad response'' (mrTRG 3, 4 and 5) in 810 (57%).

Pathological complete tumor response (pT0) was found in 293 (11.0%). Pathological T-stage was ≥T3 in 1192 (44.9%). Positive lymph nodes were detected (pN ≥ 1) in 788 (29.7%). In total, the CRM was positive in 107 (4.0%). TME specimen quality was defined as complete or near-complete in 2419 (91.2%). The composite rate for poor pathological outcome [positive resection margin, either CRM or distal resection margin (DRM), incomplete TME specimen or rectal perforation] was 224 (8.4%).

TABLE 3. Preoperative Risk Scoring for a Positive CRM Based on Prediction Model

Preoperative Risk Scoring	
Risk Factor	**Weight**
Tumor height from AJR 0 to 1 cm	1.5
Anterior tumor location	1
cT4 tumor	1.4
EMVI on baseline MRI	1.2
CRM+ on baseline MRI	1.1
Cumulative points	6.2

Note: The coefficients derived from the multivariate analysis were multiplied by 10 and used as weights in the nomogram for predicting the risk of positive CRM after TaTME for rectal cancer.

Development of the Predictive Risk Model

Table 2 shows the multivariable analysis of risk factors for positive CRM. A positive CRM after TaTME was independently associated with low tumors located within 1 cm from the ARJ, anterior tumors, cT4 tumors, EMVI on MRI and involved or threatened CRM on baseline MRI [odds ratios (ORs) 2.09, 1.66, 1.93, 1.94, and 1.72, respectively]. Resecting the sphincter by abdomino-perineal excision was just not significantly associated with CRM involvement ($P = 0.051$). No patient-related factors, such as male sex, obesity (BMI $> 30\,kg/m^2$), or previous prostatectomy, were associated with a positive CRM (Supplemental Table 1, http://links.lww.com/SLA/B727).

The weights of the individual risk factors represent the log of the odds ratios, and are shown in Table 3. The weights for the 5 risk factors were 1.5 for tumors within 1 cm from the ARJ, 1 for anterior tumors, 1.4 for cT4 tumors, 1.2 for mrEMVI positive, and 1.1 for involved or threatened CRM on baseline MRI. The nomogram, resulting from the cumulative weights, is displayed in Figure 1. When no risk factors are present (cumulative score of 0), the predicted risk of positive CRM is 1.5%. A cumulative score of 1, 2, 3, 4, 5, 6, or the maximum score of 6.2 points is correlated with a predicted positive CRM risk of 2.5%, 2.9%, 5%, 8.9%, 15.5%, 18.5%, or 27.9%, respectively.

The ROC curve was 0.715 (CI 0.669–0.703) and after correcting for optimism, the c-statistic was 0.703. The curves are shown in Supplemental Figure 1, http://links.lww.com/SLA/B727.

The model-predicted risk of a positive CRM compared with the actually observed risk of positive CRM in this cohort is displayed in Figure 2. Table 4 shows the predicted risk (and cumulative score) for pCRM involvement according to the 5 independent risk factors.

DISCUSSION

Involvement of the CRM is considered as one of the most important causes of preventable locoregional recurrence in patients undergoing surgery for rectal cancer.[4] The consequences of a locoregional relapse are significant, with a direct impact on morbidity, mortality, quality of life, and treatment costs. Therefore, given the increase in popularity and prevalence of the transanal approach in

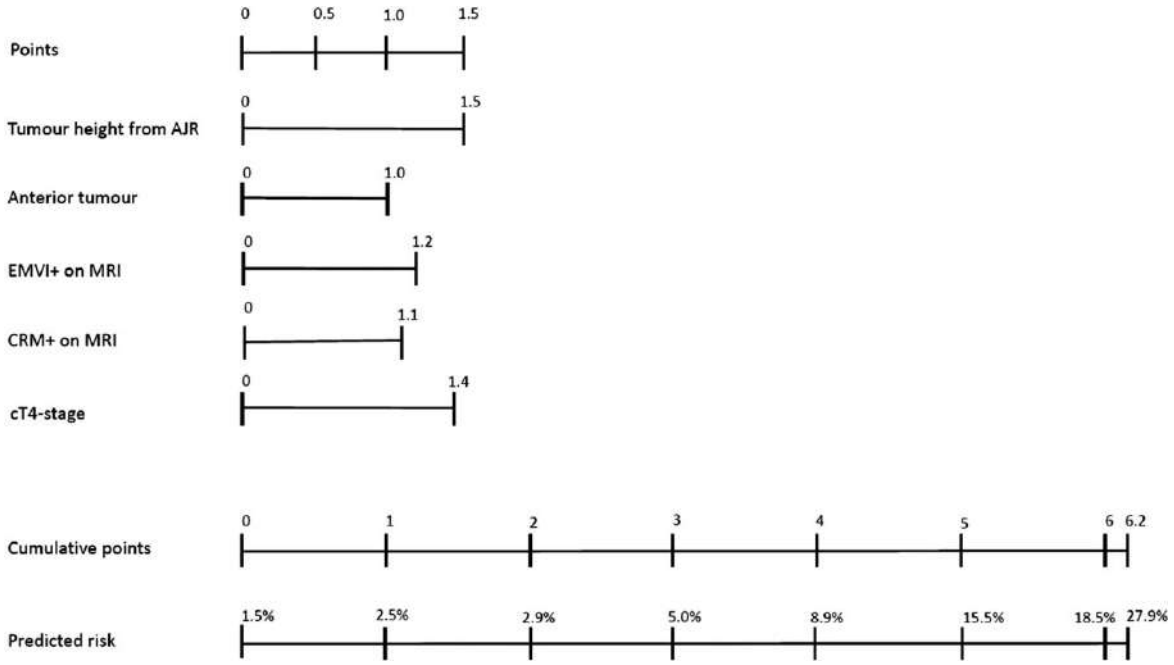

FIGURE 1. Nomogram for predicting positive CRM rate after TaTME. Note: Instructions for use: Sum the points achieved for each preoperative predictor and locate this sum on the ''cumulative points axis.'' Draw a line straight down to find the patient's probability of attaining a positive CRM.

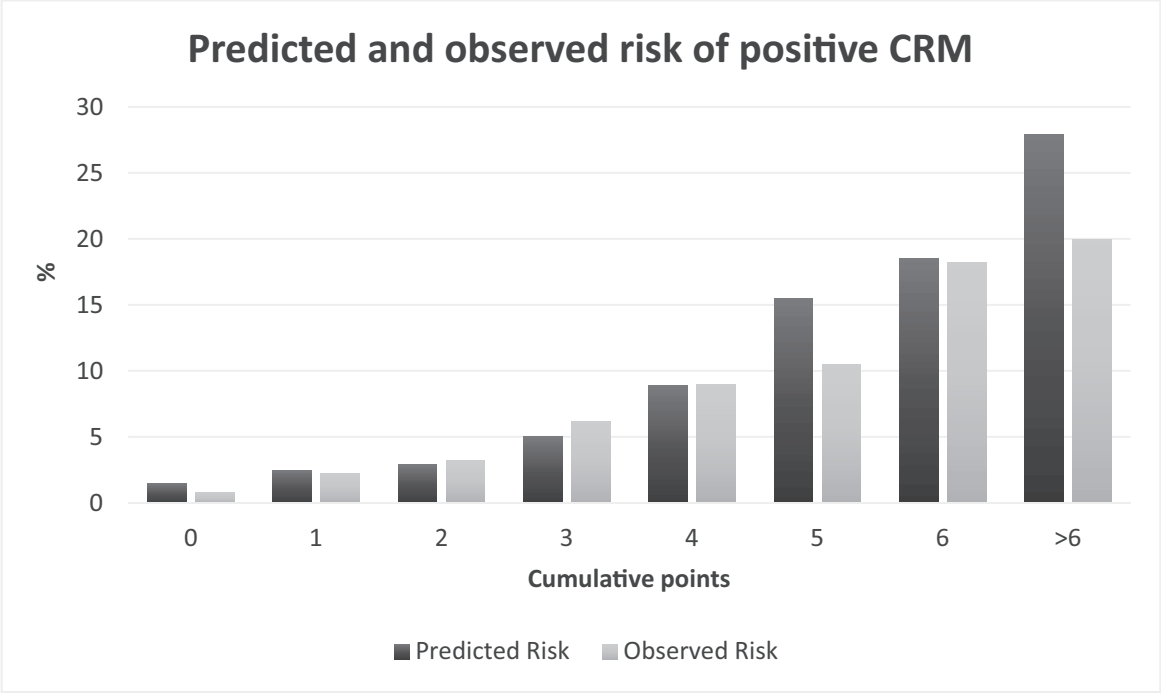

FIGURE 2. Comparison of observed and model-predicted risk of positive CRM.

TABLE 4. The Predicted Risk (and Cumulative Score) for pCRM Involvement According to the Five Independent Risk Factors

Predicted risk % of pCRM incolvement (cumulative score)									
		cT1-3-stage				cT4-stage			
EMVI on MRI	CRM on MRI	Tumor height >1cm ARJ		Tumor height ≤1cm ARJ		Tumor height >1cm ARJ		Tumor height ≤1cm ARJ	
⊖	⊖	**1.5%** (0.0)	**2.5%** (1.0)	**3.2%** (1.5)	**5.4%** (2.5)	**3.0%** (1.4)	**5.0%** (2.4)	**6.4%** (2.9)	**10.4%** (3.9)
⊖	⊕	**2.6%** (1.1)	**4.3%** (2.1)	**5.5%** (2.6)	**9.0%** (3.6)	**5.2%** (2.5)	**8.5%** (3.5)	**10.7%** (4.0)	**16.9%** (4.1)
⊕	⊖	**2.8%** (1.2)	**4.7%** (2.2)	**6.0%** (2.7)	**9.7%** (3.7)	**5.6%** (2.6)	**9.1%** (3.6)	**11.5%** (4.1)	**18.1% (5.1)**
⊕	⊕	**4.8%** (2.3)	**7.9%** (3.3)	**10.0%** (3.8)	**15.9%** (4.8)	**9.4%** (3.7)	**15.0%** (4.7)	**18.6%** (5.2)	**27.9%** (6.2)
		not Anterior	**Anterior**	**not Anterior**	**Anterior**	**not Anterior**	**Anterior**	**not Anterior**	**Anterior**

Green, low (<5%) predicted risk of pCRM positivity; Amber, intermediate (5%–15%) predicted risk of pCRM positivity; Red, high (>15%) predicted risk of pCRM positivity.

rectal cancer surgery, it was important to investigate the incidence of positive CRM and preoperative risk factors for a positive CRM after TaTME surgery. In the present study, the positive CRM rate in a large number of patients treated by TaTME was 4.0%, which can be considered as an indirect marker of good surgical oncological performance. In this study we analyzed the predictive factors for CRM involvement and noted that these were solely tumor characteristics, specifically tumors up to 1 cm from the ARJ, anterior position, cT4, and baseline MRI findings of mrEMVI positive and threatened CRM. Patient-related factors, such as male sex and BMI, which are known to pose greater technical difficulty in a conventional approach from the abdomen, did not influence CRM outcome after TaTME.

The transanal approach has been reported to enhance access to, and better visualization of, the distal part of the rectum. Thus, allowing for a more accurate oncologic dissection and increase the quality of the TME. In a randomized trial, Denost et al reported that the perineal dissection was associated with a decreased risk of CRM involvement, compared with a purely abdominal TME (18% vs 4%; $P = 0.025$).[23] The oncological superiority of the transanal approach, and more specifically TaTME, was reinforced by a recent meta-analysis that showed a higher rate of complete mesorectal resection (OR 1.75; 95% CI, 1.02–3.01; $P = 0.04$), together with a lower rate of positive CRM (OR 0.39; 95% CI, 0.17 to 0.86; $P = 0.02$).[24] In the conventional laparoscopic TME, working in the low pelvis with straight instruments may be extremely challenging, even for experienced colorectal surgeons, especially in patients with challenging anatomy. Moreover, parameters such as male sex and obesity have been associated with rates of positive CRM up to 18% to 21%.[16]

In the first report from the international TaTME registry, Penna et al reported that low tumors, positive CRM on staging MRI, and extensive abdominal dissection were independent risk factors for a poor pathological specimen.[19] The results of the present study concur with those findings, reinforcing the authors' suggestion that adverse patient characteristics, traditionally increasing the difficulty of rectal resection, are less problematic in TaTME. The MERCURY II study also reported on the predicted risk of pathological positive CRM, based on MRI findings,[25] and found the same risk factors as this study, with exclusion of cT4 tumors. These high-risk tumor features are difficult to modify, and more evidence is needed to guide the surgeon in deciding the optimal technique for each case in this high-risk group. However, TaTME seems to mitigate the effect of adverse patient-related factors, potentially improving oncological outcomes in a high-risk group.

In Table 4, the predicted risk of pCRM involvement for individual patients with different combinations of risk factors can be seen. This model provides a framework for surgeons to identify the high-risk patients (>15%) and decide preoperatively on the best surgical technique for each patient. In those cases, nonrestorative procedures or beyond TME approaches should always be considered, of course, in discussion with the patient.

In this study, the strongest predictor for CRM positivity was a tumor <1 cm from the ARJ. The ARJ in adults is located approximately 2.1 cm from the anal verge.[26] In the Mercury II study, similar analyses among patients with low rectal cancer (≤6 cm from the anal verge) identified tumor height <4 cm from the anal verge as one of the main risk factors for pathological CRM involvement (OR 3.39; 95% CI, 1.3–8.8; $P = 0.012$).[25] The association between low tumors and a higher risk of circumferential margin involvement can be explained by the tapering of the mesorectum toward the anus, thereby reducing the range for obtaining clear margins.

EMVI was also found as a prognostic factor for obtaining a positive CRM in this risk model. A systematic review by Chand et al[27] found that the presence of EMVI clearly leads to worse survival outcomes; however, there has been huge variation in the prevalence of EMVI through inconsistent reporting. They propose that as detection rates become more consistent, by standardizing histopathological definitions and the increased use of MRI, EMVI may be considered as part of risk-stratification in rectal cancer.

Although a good correlation between mrTRG status and the final histopathology has been shown,[28] we did not find mrTRG response to be significant associated with CRM status in multivariate regression analysis.[15] MRI is increasingly playing an important role in restaging rectal cancer patients after neoadjuvant treatment. However, it can be challenging to differentiate between residual tumor and fibrosis, leading to a moderate degree of heterogeneity among radiologists, which may have influenced the findings in the present study.[29]

Statistical predictive risk models and nomograms can be used to forecast oncological patient outcomes.[30] In the present study, a dataset of 2653 rectal cancer patients treated with TaTME was used to develop a model that preoperatively identifies patients at high-risk of a positive circumferential margin resection. This high-risk group

may benefit from different treatment modalities, such as prolonged neoadjuvant therapy, additional boost doses of radiotherapy, or even extended surgical resection. This predictive model may improve outcomes of TaTME, by guiding professionals in identifying high-risk patients and selecting the optimal treatment plan, reducing the chance of noncurative surgery.

This study has some limitations. First, the results are based on registry information, introducing the potential for selection bias, as well as relying on accurate recording of data. Recording cases on the registry is not obligatory, and can be very time consuming, which is why not all practicing surgeons contribute cases to the registry and it might be that some ''bad'' cases were not recorded on the registry. Second, with this novel approach, a learning curve is present and complete expertise is not achieved until several cases are performed, leading to better outcomes in surgeons with increased experience. In this article, the experience of the surgeon, learning curve, and case volume of the center were not taken into account, though they definitely influence results. This important issue will be further assessed in a future registry project, specifically focusing on learning curve for TaTME. Also, due to the design of the registry, pathological assessment was not standardized and the specimens were assessed by local pathologists. Although many pathological definitions, as TNM-staging and TME specimen quality, are standardized, this may have led to inconsistencies. Lastly, in this study, we could only perform an internal validation of the predictive model. Future studies should assess the external validity of the formulated predictive model, before definite conclusions can be drawn.

In summary, this study reports a 4% rate of positive CRM in a large cohort of patients and suggests that key predictive factors for positive CRM after TaTME were restricted to 5 tumor characteristics. CRM involvement is a strong predictor of recurrence and survival, and awareness of high risks may facilitate prevention of noncurative surgery in selected patients. Knowledge of these predictive factors will help guide patient selection, facilitate a more constructive discussion with patients regarding their risks and prognosis, and enhance a tailored treatment approach to optimize oncological outcome.

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DISCUSSANTS

Pawel Mroczkowski (Kassel, Germany):

The paper describes a predictive model for CRM-positivity after TaTME. Several issues limit the validity of the conclusions and should be clarified:

- No standardized pathology processing and reporting
- No standardized radiological processing and reporting
- No clinical validation of the data entered in the registry, which is possibly a selection bias
- Number of missing and imputed data is not given
- No standardized time period between NAT and surgery; the relationship between this gap and positive CRM was not analyzed
- Surgeon- and hospital-dependent factors were not analyzed

The concept of the registry is understandable – to achieve as much information as possible about the implementation of a new surgical technique – and the authors are to be congratulated on this. However, the price of this approach is a huge heterogeneity in the data and different oncological concepts, especially TaTME for threatened CRM, and obviously, no ''watch & wait'' option for complete response. The practical use of the proposed nomogram could be also questioned. The presented results do not have the value of a strictly conducted and controlled RCT, but do have the beauty of the real-world surgical data. Limited implementation of RCT in the clinical practice is well known. So, the results achieved by the authors should not be ignored, also inspiring the improvement of other concepts of registries, which will remain relevant for the improvement of surgical knowledge.

Response From Roel Hompes (Amsterdam, The Netherlands):

Thank you very much for your insightful comments. I believe that the first 3 comments were grouped together as they're valid and known limitations of working with registry data. When it comes to pathology reporting, of course, there is no standardized reporting of pathology, as we would have in an RCT. However, we do work with data captured under very standardized definitions, which we provided on the registry. For the pathology reporting, the most inaccurate data could come from the grading of the specimen quality, which we didn't use as a primary endpoint. We also acknowledge that there are variable definitions throughout the participating centers for a ''positive CRM.'' So, although this is a data point on the registry, we also record the distance of the primary tumor or positive lymph node toward that CRM. Therefore, instead of looking at whether the surgeon checked the ''positive CRM'' box, we actually looked at the distance toward the CRM, and determined whether it's a pathological involved margin, based on the definitions we gave in the methods section.

With regards to the radiological outcomes, I think that for surgeons working or dealing with rectal cancer, EMVI is not considered as a standard parameter for preoperative imaging. This is reflected in the registry data, with quite a large proportion of missing data on this variable. There is a recent systematic review by Chand et al (*World J Gastroenterol*, 2016), which has shown that

there is quite a lot of variability in reporting EMVI. So, I think that this is definitely a point for future improvement.

Concerning the selection bias, again, this is not a randomized control trial. It's registry data, where we have to acknowledge, that we can't be certain that all surgeons also included their worst cases. However, we have made every effort to make the data as accurate as possible. It took us 6 months to clean the data. We went through each case, looked at any inconsistencies and missing data, and then emailed each surgeon individually with these queries. At the end of the day, I would like to make the point that this is real-world data and the best data we have so far.

With regards to the data imputation and the missing data, as mentioned, we tried to limit the amount of missing data by contacting surgeons individually. Still, we have used the single imputation and the predictive mean matching for missing data, as discussed with our statistician within our department. We've acknowledged that multiple imputations would be better. However, that would lead us to doing a single imputation ten times, which would mean that we would get ten different datasets and models, and we would struggle to see how we would combine these models. That's why we chose a single imputation, and the range of the data that was imputed was from 1% to 30%; the 30% was particularly relevant for the EMVI data. I agree that 30% appears high, but simulation studies have shown that even if data is imputed up to 80%, one can still get valid prognostic models.

With regards to your question about the interval period between neo-adjuvant therapy (NAT) and surgery, it's again registry-based data, and not standardized. What we saw was a median interval between neoadjuvant treatment and the surgical procedure of 9.5 weeks [IQR 7.7–12.0]. When you look at the literature, I believe that this is an acceptable interval to achieve downstaging. We did analyze whether there is a difference in getting a pathological involved margin, and no relationship was found between a longer waiting time and positive CRM rate (4.2% in $<$8 wk interval vs 4.5% in $>$8 wk interval, $P = 0.815$).

Ultimately, your last question is also very valid. We do have data on the volume of surgeons. This is part of another project that we're doing based on this dataset, which aims to determine the learning curve for various endpoints. We don't have accurate data on hospital volume because we don't know what the denominator (total number of procedures for rectal cancer) is for each individual unit. However, we have a new project based on Dutch population-based data in the pipeline, as this allows us to not only have data on the exact number of TaTME procedure performed, but also on the denominator of total procedures of rectal cancer.

Finally, I think your comments have strengthened insight into the paper, and will, hopefully, improve the overall message of the paper.

Ronan P. O'Connell (Dublin, Ireland):

Thank you for presenting these data. You say that they are real-world data and they are real-world data in the registry. However, is the "real world" in the real world? That is really one of the concerning things because there is a substantial learning curve to this operation. Many of us have spent our lives learning how to do TaTME properly from above, and now, people are beginning to try to learn the anatomy from completely the opposite end. It is difficult and there are complications that we are seeing with this technique, which we don't generally see with doing it from above. So, the first point is that you say it's "real world," but is it really "real world"?

The second point is that you have said that patient factors, such as sex or obesity, did not come through as being statistically important, and yet, you state in your introduction that these are selected patients. You have a greater number of men and obese patients. So, how can you then deduce that this is not something that is relevant?

Response From Roel Hompes (Amsterdam, The Netherlands):

I do think that it's the "real world." Within population-based datasets, we do observe that surgeons tend to select the most difficult patients for TaTME. I think that this is an issue we need to address, particularly within the learning curve. We have published data on how TaTME was implemented in the Netherlands (Detering et al, *J Am Coll Surg*, 2019), and there you can clearly see that surgeons in the learning curve are choosing the most difficult patients. This leads to more morbidity, more anastomotic leaks and longer hospital stays. So, I think that this model can give them an idea of which patients should ideally not be chosen within their learning curve, even though they might be the ideal candidates for TaTME.

With regards to your second question, if patient factors, such as sex and obesity, were to be related, I would expect that it would have come through in the analysis. Both of these factors weren't even significant in the univariate analysis. Of course, they are relevant in that they comprise the cases, where one expects to gain the most benefit from the technique.