

**Immunopathological features of early age cancer: microsatellite
instability in colorectal cancer**

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Doctor of Philosophy**

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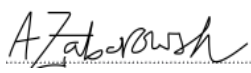
Declaration

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Summary

This thesis comprises clinical and laboratory-based research relating to the immunobiological features of colorectal cancer. The first component describes the immunopathological features and molecular characteristics of early age onset colorectal cancer (defined as diagnosis before the age of 50) with a specific focus on microsatellite instability. A comprehensive review of the literature was performed and is presented in Chapter 1. Institutional data from St Vincent's University Hospital, Dublin was analysed to compare disease-specific outcomes of patients diagnosed with rectal cancer aged less than 50 to patients aged over 50 (Chapter 3). In summary, young patients with rectal cancer were more likely to receive neoadjuvant and adjuvant treatment compared to older counterparts, yet displayed comparable disease-specific outcomes. Microsatellite instability and a diagnosis of Lynch Syndrome were more common in early age onset disease.

As early age onset colorectal cancer is a relatively rare phenomenon, an international collaborative group was established to aggregate large volume data. In a study of over 3500 patients (Chapter 4), young patients present with advanced disease yet survival outcomes are comparable to those of stage-matched older patients. The impact of molecular characteristics, specifically microsatellite status, on oncological outcomes in colon and rectal cancer is presented in Chapter 5 and 6 respectively. In contrast to late-onset colon cancer, microsatellite instability in young adults was not associated with reduced likelihood of nodal positivity. In patients with rectal cancer,

microsatellite instability was associated with an increased complete pathological response rate. Patients with microsatellite instability demonstrated better survival across all disease stages in both colon and rectal cancer (although not statistically significant).

The second component of this thesis describes the immune landscape of colorectal cancer at cellular level. Tumour and healthy colon samples of patients with treatment naïve non-metastatic colorectal cancer undergoing surgery were analysed by flow cytometry and single cell RNA sequencing. The focus of Chapter 7 is expression of inhibitory checkpoints on tumour infiltrating lymphocytes. As the studies described in Chapter 5 and 6 evaluated the clinical impact of microsatellite instability, we sought to investigate the effect of microsatellite instability at immune cell level, specifically inhibitory checkpoint expression, using flow cytometry. In summary, PD-1 expression was significantly increased in tumours with microsatellite instability but there was significant variation among patients with the same microsatellite status.

The study outlined in Chapter 8 evaluates the functional properties of $\gamma\delta$ T cells in colon cancer. Flow cytometry and single cell RNA sequencing identified functionally distinct subpopulations of V δ 1 cells in the tumour. One subset produced IFN γ , demonstrating a cytotoxic phenotype. The other subset demonstrated a wound healing phenotype, producing amphiregulin.

Table of Contents	i
Index of Figures	viii
Index of Tables	xii
Acknowledgments	xiv
Publications	xvi
Presentations	xx
Abbreviations	xxii
Chapter 1. Introduction	1
1.1 Colorectal Cancer	2
1.1.1 Epidemiology	3
1.1.2 Pathogenesis and molecular profile	5
1.1.3 Clinical management of colorectal cancer	10
1.2 Early age onset colorectal cancer	27
1.2.1 Definition and epidemiology	27
1.2.2 Clinical and pathological features	28
1.2.3 Molecular profile	29
1.2.4 Hereditary disease	32
1.2.5 Oncological outcomes	34
1.3 Cancer and the immune response	36
1.3.1 Immunoediting	36
1.3.2 The immune microenvironment	37
1.3.3 Tumour biology and the immune response	43
1.3.4 Multimodal therapy and the immune response	45
1.4 Tumoral immunogenicity and immunotherapy	50
1.4.1 Intra-tumoral immune response	50
1.4.2 Inhibitory checkpoint therapy	50

1.4.3	Checkpoint therapy in colorectal cancer	52
1.5	Hypothesis and Aim	54
Chapter 2.	Materials and Methods	55
2.1	Patient cohort	56
2.2	Clinical pathway	57
2.2.1	Neoadjuvant therapy	57
2.2.2	Surgery	57
2.2.3	Perioperative protocol	58
2.2.4	Adjuvant chemotherapy	58
2.2.5	Follow up	58
2.3	Histopathology	59
2.4	Molecular profile	60
2.4.1	Mismatch repair status	60
2.5	Flow cytometric analysis	61
2.5.1	Materials	63
2.5.2	Study cohort	64
2.5.3	Samples collected	64
2.5.4	Sample transfer and storage	64
2.5.5	Sample processing	66
2.5.6	Data analysis	70
2.6	Single cell RNA sequencing	72
2.7	Data collection and management	74
2.8	Data analysis	78

Chapter 3. Clinicopathological features and oncological outcomes of patients with early age onset rectal cancer	79
Abstract	80
3.1 Background	81
3.2 Methods	82
3.2.1 Study population	82
3.2.2 Treatment protocol	82
3.2.3 Pathological analysis	83
3.2.4 Study endpoints	84
3.2.5 Statistical analysis	84
3.3 Results	85
3.3.1 Patient demographics, clinical characteristics and pathological features	85
3.3.2 Survival	87
3.3.3 Disease recurrence	88
3.3.4 Univariable and multivariable analyses of factors that influence survival	89
3.4 Discussion	90
 Chapter 4. An international multicentre study of clinical characteristics, pathological features and oncological outcomes of patients with early age onset colorectal cancer	 94
Abstract	95
4.1 Background	97
4.2 Methods	99
4.2.1 Study participants	99
4.2.2 Data collection	99

4.2.3 Treatment	100
4.2.4 Pathology	100
4.2.5 Study endpoints	101
4.2.6 Statistical analysis	101
4.3 Results	103
4.3.1 Baseline demographics	103
4.3.2 Clinical characteristics	103
4.3.3 Neoadjuvant and adjuvant therapy	105
4.3.4 Pathology	105
4.3.5 Molecular features	106
4.3.6 Oncological outcomes	106
4.3.7 Factors influencing survival	107
4.4 Discussion	110
 Chapter 5. Comparing disease-specific outcomes of patients with early age onset colon cancer according to microsatellite status	 115
Abstract	116
5.1 Background	117
5.2 Methods	119
5.2.1 Study participants	119
5.2.2 Data collection	119
5.2.3 Study endpoints	120
5.2.4 Statistical analysis	120
5.3 Results	121
5.3.1 Baseline demographics	121
5.3.2 Pathological features	122
5.3.3 Molecular characteristics	123

5.3.4 Survival	123
5.3.5 Disease recurrence	127
5.3.6 Factors predictive of disease-specific outcomes	127
5.3.7 Comparison of sporadic MSI tumours and MSI tumours arising in the context of a hereditary cancer syndrome	129
5.4 Discussion	131
 Chapter 6: The impact of MSI on treatment response and oncological outcomes in young patients with rectal cancer	 134
Abstract	135
6.1 Background	136
6.2 Methods	138
6.2.1 Study participants	138
6.2.2 Data collection	138
6.2.3 Study endpoints	139
6.2.4 Statistical analysis	139
6.3 Results	140
6.3.1 Baseline demographics	140
6.3.2 Pathological features	141
6.3.3 Molecular characteristics	142
6.3.4 Oncological outcomes	142
6.3.5 Factors predictive of disease-specific outcomes	145
6.4 Discussion	147
 Chapter 7. Uncoupling of tumour-infiltrating lymphocyte checkpoint expression and microsatellite instability status in colorectal cancer	 153

Abstract	154
7.1 Background	156
7.2 Methods	160
7.2.1 Study population	160
7.2.2 Pathological analysis	160
7.2.3 Immunophenotyping	161
7.2.4 Statistical analysis	162
7.3 Results	163
7.3.1 Baseline demographics and clinical data	163
7.3.2 Microsatellite instability status	163
7.3.3 Inhibitory checkpoint expression	164
7.3.4 Functional analysis of TILs	183
7.3.5 Association between PD-1 expression and clinicopathological parameters	193
7.4 Discussion	195
 Chapter 8. Functionally distinct $\gamma\delta$ T cells in colorectal cancer	 202
Abstract	203
8.1 Background	204
8.2 Methods	206
8.2.1 Study population	206
8.2.2 Pathological analysis	206
8.2.3 Immunophenotyping	206
8.2.4 Statistical analysis	207

8.3 Results	
8.3.1 Enrichment of V δ 1 cells in tumour compared to healthy colon	208
8.3.2 Inhibitory checkpoint expression and cytotoxicity of intra-tumoral $\gamma\delta$ T cells	209
8.3.3 Functional heterogeneity of intra-tumoral $\gamma\delta$ T cells	212
8.3.4 $\gamma\delta$ T cell expression of AREG in healthy colon	214
8.3.5 AREG expression is associated with NKp80+ V δ 1 cells	216
8.4 Discussion	219
 Chapter 9: Discussion and conclusion	 223
9.1 Discussion	223
9.1.1 Summary of key findings	223
9.1.2 Limitations	230
9.1.3 Future perspectives	237
9.2 Conclusion	246
References	247

Index of Figures

Figure 1. The immunomodulatory effects of surgery, chemotherapy, and radiotherapy.....	47
Figure 2. Activation of CD8+ T cells in the tumour microenvironment and mechanisms of inhibitory checkpoint blockade.....	51
Figure 3. REACCT Collaborative logo.....	76
Figure 4. Geographical representation of participating institutions of the REACCT Collaborative.....	77
Figure 5. Kaplan-Meier curve of overall survival for patients with rectal cancer aged less than 50 years vs. aged 50 years and above.....	87
Figure 6. Kaplan-Meier curve of disease-free survival for patients with rectal cancer aged less than 50 years vs. aged 50 years and above.....	88
Figure 7. Kaplan-Meier curve of overall survival for patients with Stage I colon cancer according to microsatellite status.....	124
Figure 8. Kaplan-Meier curve of disease free survival for patients with Stage I colon cancer according to microsatellite status.....	124
Figure 9. Kaplan-Meier curve of overall survival for patients with Stage II colon cancer according to microsatellite status.....	125

Figure 10. Kaplan-Meier curve of disease free survival for patients with Stage II colon cancer according to microsatellite status.....	125
Figure 11. Kaplan-Meier curve of overall survival for patients with Stage III colon cancer according to microsatellite status.....	126
Figure 12. Kaplan-Meier curve of disease free survival for patients with Stage III colon cancer according to microsatellite status.....	126
Figure 13. Kaplan-Meier curve of overall survival for patients with Stage I-III rectal cancer according to microsatellite status.....	143
Figure 14. Kaplan-Meier curve of disease free survival for patients with Stage I-III rectal cancer according to microsatellite status.....	144
Figure 15. Kaplan-Meier curve of disease free survival for patients with Stage I (A), II (B) and III (C) rectal cancer according to microsatellite status.....	145
Figure 16. Percentage of CD3+ cells expressing PD-1 and TIGIT in uninvolved healthy colonic tissue and tumour.....	166
Figure 17. Percentage of CD3+ cells expressing PD-1 and TIGIT in paired uninvolved healthy colonic tissue and tumour samples.....	167
Figure 18. Percentage of CD3+ cells expressing PD-1 in uninvolved healthy colonic tissue and	

tumour.....	168
Figure 19. Single cell RNA sequencing showing subsets of tumour-infiltrating lymphocytes and inhibitory checkpoint expression.....	170
Figure 20. Single cell RNA sequencing showing TIL subsets and inhibitory checkpoint expression.....	172
Figure 21. PD-1 and TIGIT expression on CD3+ subsets in tumour and healthy colon.....	174
Figure 22. CTLA4 expression on CD3+ subsets in tumour and healthy colon.....	175
Figure 23. LAG3 and TIM3 expression on CD3+ subsets in tumour and healthy colon.....	176
Figure 24. Percentage of CD3+ cells expressing PD-1 in uninvolved healthy colonic tissue and tumour according to MSI status.....	179
Figure 25. Representative flow plot of CD3+ cells in tumour expressing PD-1.....	180
Figure 26. Percentage of CD3+ cells expressing TIGIT in uninvolved healthy colonic tissue and tumour according to MSI status.....	181
Figure 27. Percentage of CD3+ cells expressing PD-1 and TIGIT in paired uninvolved healthy colonic tissue and tumour.....	182
Figure 28. Patients with MSI tumours have increased PD-1 expression on intra-tumoral T cells.....	183

Figure 29. Percentage of CD3+ cells in uninvolved healthy colon and tumour producing IFNγ.....	185
Figure 30. Tumoral CD3+ cell production of IFNγ on the basis of PD-1 and TIGIT expression.....	186
Figure 31. Percentage of CD3+ cells in uninvolved healthy colon and tumour producing IFNγ according to MSI status.....	187
Figure 32. Percentage of CD3+ cells in the tumour producing IL-17a and amphiregulin.....	188
Figure 33. Percentage of CD3+ cells in the tumour producing IL-17a according to MSI status.....	189
Figure 34. Percentage of CD3+ cells in the tumour producing AREG according to MSI status.....	190
Figure 35. Single cell RNA sequencing of colon tumours showing subsets of TILs.....	191
Figure 36. Single cell RNA sequencing of colon tumours showing subsets of TILs.....	192
Figure 37. Vδ1 T cells in healthy colon and colon tumours.....	209
Figure 38. $\gamma\delta$ T cell immune checkpoint expression.....	211
Figure 39. Functionally distinct clusters of $\gamma\delta$ T cells in colon tumours.....	214
Figure 40. T cell production of amphiregulin in colon tumour and healthy colon.....	215

Figure 41. $\gamma\delta$ T cell expression of NKp80 & TIGIT in colon tumour.....	217
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Figure 42. Age-based comparison of MSI colon cancer.....	226
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Index of Tables

Table 1. American Joint Committee on Cancer staging of colorectal cancer 8th Edition.....	13
Table 2. Pathological features and molecular profile of early age onset colorectal cancer.....	32
Table 3. Flow cytometry antibodies and stains.....	63
Table 4. Comparison of demographics and clinicopathological features according to age.....	86
Table 5. Age-based Univariable Logistic Regression of Factors Predicting Disease Specific Survival.....	89
Table 6. Demographics and clinicopathological data of patients with colonic and rectal cancer.....	104
Table 7. Neoadjuvant and adjuvant therapy according to disease stage.....	105
Table 8. Univariable and multivariable logistic regression of factors predicting disease free survival in patients with colonic cancer stage I-III.....	107
Table 9. Univariable and multivariable logistic regression of factors predicting disease free survival in patients with rectal cancer stage I-III.....	108
Table 10. Comparison of demographics and clinicopathological data according to microsatellite status in patients with colonic cancer.....	122

Table 11. Univariable Logistic Regression of Factors Predicting Disease Free Survival for MSI colon cancers.....	128
Table 12. Univariable Logistic Regression of Factors Predicting Disease Free Survival for MSS colon cancers.....	129
Table 13. Comparison of demographics and clinicopathological data according to microsatellite status in patients with rectal cancer.....	141
Table 14. Comparison of pathological features according to microsatellite status in patients with rectal cancer.....	142
Table 15. Univariable Logistic Regression of Factors Predicting Disease Recurrence for MSI rectal cancers.....	146
Table 16. Univariable Logistic Regression of Factors Predicting Disease Recurrence for MSS rectal cancers.....	146
Table 17. Baseline demographics and clinicopathological features of study cohort.....	164
Table 18. Comparison of clinicopathological features according to PD-1 expression.....	194
Table 19. Age-based comparison of MSI rectal cancer.....	227

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Published chapters

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2. Zaborowski, A.M, REACCT Collaborative. Characteristics of Early-Onset vs. Late-Onset Colorectal Cancer: A Review. ***JAMA Surg.*** 2021;156(9):865–874. (Chapter 1)
3. Zaborowski A.M, Murphy B, Creavin B, et al.
Clinicopathological features and oncological outcomes of patients with young-onset rectal cancer. ***Br J Surg.*** Apr 2020;107(5):606-612 (Chapter 3)
4. Zaborowski A.M, REACCT Collaborative. Impact of microsatellite status in early-onset colonic cancer. ***Br J Surg.*** 2022 Jun 14;109(7):632-636 (Chapter 5)
5. Zaborowski A.M, REACCT Collaborative. Microsatellite instability in young patients with rectal cancer: molecular findings and treatment response. ***Br J Surg*** 2022 Feb 24;109(3):251-255 (Chapter 6)

6. Zaborowski AM, REACCT Collaborative. Colorectal cancer in the young: Research in Early Age Colorectal Cancer Trends (REACCT) Collaborative. **Cancers** 2023 May 30;15(11):2979 (Chapter 4)
7. Harmon C, Zaborowski A, et al. The microenvironment of colorectal cancer induces $\gamma\delta$ T cell dichotomy with opposing cytotoxic and wound healing functions. **Nat Cancer** 2023 July 20 (Chapter 8) *Online ahead of print*

Other publications

Book chapters

1. Neoadjuvant Therapy for Locally Advanced Rectal Cancer. Surgical Management of Advanced Pelvic Cancer. Wiley 2021
2. Surgical Management of Polyposis Syndromes. Hereditary Colorectal Cancer. Cambridge Scholars Publishing 2022
3. Predicting Pathological Response to Neoadjuvant Chemoradiotherapy. Locally Advanced Rectal Cancer. Wiley (*in press*).
4. Oncotherapeutic Challenges in Early Age Cancer. Cancer and Immunology Handbook. Springer 2023

Peer reviewed articles unrelated to thesis

1. Zaborowski A, Stakelum A, Winter DC. A Systematic Review of Outcomes following Total Neoadjuvant Therapy for Locally Advanced Rectal Cancer. *Br J Surg* 2019 July;106 (8), 979-987.
2. Stakelum A, Zaborowski A, Collins D, Winter DC. The Influence of the Gastrointestinal Microbiome on Colorectal Metastasis: A Narrative Review. *Colorectal Dis.* 2020 Sep;22(9):1101-1107.
3. Patel SV, Hendren S, Zaborowski A, Winter D. Evidence-based Reviews in Surgery Long-term Outcome of Surgery Versus Conservative Management for Recurrent and Ongoing Complaints After an Episode of Diverticulitis: Five-year Follow-up Results of a Multicenter Randomized Controlled Trial (DIRECT-Trial). *Ann Surg.* 2020 Aug; 272(2):284-287.
4. Zaborowski A, Heneghan HM, Fiore B, Stafford A, Gallagher T, Geoghegan J, Maguire D, Hoti E Neoadjuvant Chemoradiotherapy and Liver Transplantation for Unresectable Hilar Cholangiocarcinoma: The Irish Experience of the Mayo Protocol. *Transplantation* 2020 Oct;104(10):2097-2104

5. Musilova J, Moran B, Sweeney CM, Malara A, Zaborowski A, Hughes R, Winter DC, Fletcher JM, Kirby B. Enrichment of Plasma Cells in the Peripheral Blood and Skin of Patients with Hidradenitis Suppurativa. *J Invest Dermatol* 2020 May;140(5):1091-1094

6. Zaborowski AM, Winter DC. Evidence-based treatment strategies for acute diverticulitis. *Int J Colorectal Dis* 2021 Mar; 36(3):467-475.

7. Zaborowski AM, Stakelum A, Winter DC. Anastomotic leak risk in complete responders to neoadjuvant therapy for rectal cancer: a systematic review. *Int J Colorectal Dis* 2021 Apr;36(4):671-676.

8. BJS Commission on surgery and perioperative care post-COVID-19. *Br J Surg* 2021 Oct 23;108(10):1162-1180. 9.

9. REACCT Collaborative. Post-operative Functional Outcomes in Early Age Onset Rectal Cancer. *Front Oncol.* 2022 May 30;12:868359

10. Zaborowski AM, Prichard RS. Adrenalectomy for metastases. *Br J Surg* 2022 Oct 14;109(11):1030-1031

Editorial experience

Junior guest editor of two e-books for Frontiers in Oncology on Locally Advanced Rectal Cancer (Volume 1 2020/21 and Volume 2 2021/22)

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Abbreviations

%	Percentage
°C	Degrees Celsius
5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
APR	Abdominoperineal Resection
ASA	American Society of Anaesthesiologists
BSA	Bovine Serum Albumin
BMI	Body Mass Index
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B1
CAPOX	Capecitabine and Oxaliplatin
CEA	Carcinoembryonic Antigen
CHEK2	Checkpoint Kinase 2
CI	Confidence Interval
CIMP	CpG island methylator phenotype
CMS	Consensus Molecular Subtypes
CRC	Colorectal Cancer
CRIS	Colorectal Cancer Intrinsic Subtypes
CT	Computed Tomography
DFS	Disease-Free-Survival
dMMR	Deficient Mismatch Repair
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
EOCRC	Early Age Onset Colorectal Cancer
EORTC	European Organisation for Research and Treatment of Cancer
FCS	Fetal Calf Serum
FDR	First Degree Relative
FOLFOX	Fluorouracil, Leucovorin, and Oxaliplatin
FSP	Frameshift Peptides
Gy	Gray
HBSS	Hanks Balanced Salt Solution
HCS	Hereditary Cancer Syndrome

HR	Hazard Ratio
HR-QL	Health-Related Quality of Life
IBD	Inflammatory Bowel Disease
IFN γ	Interferon gamma
IHC	Immunohistochemistry
ILCs	Innate Lymphocytes
IL-17	Interleukin-17
KRAS	Kirsten Rat Sarcoma
LAG-3	Lymphocyte-Activation Gene 3
LARC	Locally Advanced Rectal Cancer
LPND	Lateral Pelvic Node Dissection
LS	Lynch Syndrome
MLH1	MutL Homolog 1
MHC	Major Histocompatibility Complex
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite instability
MSS	Microsatellite stable
N	Number
nCRT	Neoadjuvant Chemoradiotherapy
NK	Natural Killer
NS	Not Significant
OR	Odds Ratio
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pCR	Pathological Complete Response
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein-1
PDX	Patient-Derived Xenografts
pMMR	Proficient Mismatch Repair
PRISM	GraphPad Prism version 7.0
P value	P value

R0	Complete Resection
RNA	Ribonucleic Acid
RPMI	Roswel Memorial Park Institute media
RT	Radiotherapy
SCRT	Short-Course Radiotherapy
SEM	Standard Error of the Mean
SMAD4	Suppressor of Mothers Against Decapentaplegic 4
SPSS	Stastical Package for the Social Sciences
TAMIS	Transanal Minimally Invasive Surgery
TEMS	Transanal Endoscopic Microsurgery
TGF- β	Transforming Growth Factor-beta
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIM3	T-cell immunoglobulin (Ig) mucin 3
TME	Tumour Microenvironment
TNF α	Tumour Necrosis Factor alpha
TNM	Tumour, Node, Metastasis
TNT	Total Neoadjuvant Therapy
TP53	Tumour Protein 53
TRG	Tumour Regression Grade
US	Ultrasound
WNT	Wingless-related integrated site

Chapter 1

Introduction

1.1 Colorectal Cancer

Colorectal cancer (CRC) is a major health problem. It accounts for 10% of all cancers diagnosed and represents the second leading cause of cancer-related death globally^{1,2}. Risk factors include increasing age and family history. Over half of cases however, are attributed to modifiable risk factors such as obesity, diet, smoking and alcohol consumption³. Approximately 40% of patients are diagnosed with early disease (stage I-II), 40% with locally advanced disease (stage III) and 20% with metastatic disease (stage IV). The most important predictor of survival is stage at diagnosis with 5-year relative survival ranging from 90% for early disease to 14% for metastatic disease⁴. Rectal cancer is more frequently localized at diagnosis than colon cancer, presumably to the earlier development of symptoms.

Despite advances in neoadjuvant/adjuvant chemotherapy and radiotherapy, surgery remains the cornerstone of curative treatment. Distinction between tumours in the colon and rectum is imperative as therapeutic strategies for each vary. Anatomically, the true rectum begins at the point of fusion of the anti-mesenteric taenia and continues to the anal verge. The surgical definition describes the rectum as beginning at the sacral promontory. In general, rectal cancer is defined as a tumour located within 15cm from the anal verge on rigid sigmoidoscopy. Radiotherapy plays an important role in the management of rectal cancer, but is not appropriate for colon cancer. Internationally standardised definitions are not only critical for clinical decision making, but also to evaluate outcomes and establish

modern benchmarks.

1.1.1 Epidemiology

CRC represents the most common intra-abdominal malignancy and is the third most commonly diagnosed cancer among both men and women in the worldwide¹. Rates vary geographically, and by race and ethnicity. The highest incidence is in the developed world with a cumulative lifetime risk of 5%. Among the 5 major racial/ethnic groups, black individuals are most affected. Reasons for geographical and racial disparity are complex, and likely relate to differences in lifestyle factors, socioeconomic status, and access to healthcare⁵. Individuals with socioeconomic deprivation are 40% more likely to develop CRC than those with high socioeconomic status, with almost half of this disparity attributable to modifiable risk factors and poor uptake of screening⁶.

Primarily a disease of the elderly, the incidence of CRC peaks in seventh and eight decades of life. In most countries, incidence and mortality is higher in men than women, particularly for rectal tumours⁷. The sex disparity also varies by age. Whilst incidence is comparable under the age of 45, it is up to 50% higher in men than women aged 55 to 74 years⁴. Although the implementation of population-based screening has led to a decrease or stabilisation in overall incidence and mortality in many countries, there has been an alarming rise in incidence in adults aged less than 50 years with the greatest annual percentage change observed in adults aged 20-39

years⁸. Based on current data from large European and North American registry studies, it is estimated that within the next decade 1 in 10 colon cancers and 1 in 4 rectal cancers will be diagnosed in individuals younger than 50 years^{8,9}. The reasons for this disproportionate increase are unknown. Environmental and lifestyle factors alone do not explain the observed trends as they are not age-related.

Epidemiological studies have consistently shown increasing age and male sex as risk factors for development of CRC¹⁰. Modifiable lifestyle factors associated with increased risk include obesity, excess alcohol consumption, cigarette smoking, a westernised diet (high in saturated fat, rich in red meat and low in fibre), and specific bacterial species, such as *Fusobacterium nucleatum* and *Bacteroides fragilis*, in the colonic microbiota¹¹⁻¹⁵. Positive family history is a known risk factor for CRC with an estimated 35% of cases due to inherited factors¹⁶. Several genome-wide association studies of CRC have successfully identified cancer susceptibility genes (such as common single-nucleotide polymorphisms) that are associated with CRC risk¹⁷⁻¹⁹. The inheritability of these genes however remains unclear. A defined hereditary cancer syndrome is present in a subgroup (approximately 5-7%) of patients with CRC²⁰. These hereditary CRC syndromes can be subdivided as non-polyposis (Lynch syndrome) and polyposis syndromes associated with varying lifetime risks.

1.1.2 Pathogenesis and molecular profile

Colorectal cancer is a disease with variable clinical course, response to therapy, and prognosis. The underpinning tumour biology is complex and displays considerable heterogeneity as it develops via several distinct oncogenic pathways. A multistep genetic model was first proposed by Fearon and Vogelstein in the 1990s²¹. This model describes a series of somatic genetic alterations that lead to well-characterised histopathological changes termed the adenoma-carcinoma sequence. At least four sequential genetic changes or ‘hits’ are needed to occur to ensure development of CRC. Although genome-wide sequencing has identified many genes implicated in colorectal carcinogenesis, the main drivers are the Kirsten Rat Sarcoma (*KRAS*) oncogene, and tumour-suppressor genes adenomatous polyposis coli (*APC*), Suppressor of Mothers Against Decapentaplegic 4 (*SMAD4*) and Tumour Protein 53 (*TP53*).

The majority of sporadic CRCs (70-90%) arise as a result of the adenoma-carcinoma pathway whereby a precursor lesion (polyp) transforms into a tumour over an estimated period of 10 to 15 years. Mutations in the *APC* gene, the earliest genetic alteration in the adenoma-carcinoma sequence, result in aberrant activation of Wnt/ β -catenin signalling and dysregulation of cellular proliferation, differentiation, and apoptosis²². Acquired activating *KRAS* mutations subsequently lead to dysplasia (loss of normal cellular morphology). Notably, *KRAS* mutations are only found in 50% of sporadic colorectal adenomas and carcinomas, suggesting that unknown

oncogenes or epigenetic mutations may also promote *APC*-driven carcinogenesis^{23,24}. Finally, loss of heterozygosity in *SMAD4* and *TP53* result in the transformation to adenocarcinoma. Approximately 10-20% of CRCs develop via the serrated neoplasia pathway (traditional or sessile serrated polyps) which is associated with mutations of the *KRAS* and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) genes, and methylation of the CpG island methylator phenotype (CIMP) gene. Germline *APC* mutations give rise to the autosomal dominant syndrome Familial Adenomatous Polyposis of which the lifetime risk of CRC is 100% without prophylactic surgery to remove all of the at risk colon and rectum.

Three forms of genetic instability have been described in CRC: chromosomal instability (CIN), microsatellite instability (MSI) and CIMP. CIN, the hallmark of most CRCs (85%), is characterised by changes in chromosome number and structure leading to an imbalance in chromosome number, sub-chromosomal genomic amplifications, and loss of heterozygosity²⁵. Alteration in genes that regulate cell proliferation or cell cycle checkpoints in turn activate pathways integral to initiation and progression of carcinogenesis. MSI is the result of defective DNA mismatch repair (MMR). Microsatellites are repetitive DNA sequences consisting of tandem repeats. A high frequency of replication errors results in the accumulation of deletion/insertion mutations at coding microsatellites. These mutations inactivate tumour suppressor genes contributing to tumorigenesis. The defect in MMR may be caused by sporadic events (e.g. epigenetic

silencing of the MLH1 gene) or by constitutive mutations in one of the MMR genes - the most common of which are MLH1, MSH2, MSH6 and PMS2 - resulting in the most common hereditary cancer syndrome (Lynch Syndrome, formerly known as hereditary non-polyposis colorectal cancer syndrome; HNPCC)²⁶. CIMP is characterized by aberrant DNA methylation at promoter CpG islands of tumour suppressor genes including *CDKN2A*, *MGMT*, and *MLH1*. Promoter methylation is a distinguishing feature of the serrated neoplasia pathway, and accounts for approximately 17% of CRCs²⁷. Clinical features of CIMP are similar to MSI-associated tumours, and typically arise in the proximal colon in elderly females²⁸. Notably, tumours can occasionally exhibit features of several pathways. For example, up to 33% of CIMP-positive tumours can display a high degree of chromosomal aberrations²⁹.

Increased understanding of the biomolecular processes has enabled the molecular stratification of patients with CRC and the development of targeted therapy against these pro-oncogenic signalling pathways. Using gene expression analysis, CRC has more recently been further subdivided into four consensus molecular subtypes (CMS) on the basis of distinguishing clinical behaviour, underpinning biology and molecular characteristics: (1) CMS1 (14%): MSI immune; favourable clinical outcome in early stage disease, adverse prognosis in the metastatic setting; (2) CMS2 '*canonical*' (37%) : epithelial gene expression profile, WNT and MYC activation, intermediate prognosis; (3) CMS3 '*metabolic*' (13%): metabolic dysregulation and KRAS

mutations, intermediate prognosis; and (4) CMS4 '*mesenchymal*' (23%): marked stromal infiltration, Transforming Growth Factor- β (TGF- β) activation and angiogenesis, poor prognosis³⁰. The remaining 13% demonstrated mixed features and were described as unclassified rather than a 5th subtype. Associations between CMS groups and clinical features were also observed. CMS1 tumours were more common in females with right-sided lesions, and more likely to exhibit a higher histological grade. CMS2 tumours were mostly left-sided, while CMS4 tumours tended to be diagnosed at advanced disease stage (stage III or IV).

Analysis of CRC patient-derived xenografts (PDXs) identified 5 subtypes on the basis of cancer cell intrinsic transcriptional features³¹. In contrast to the tissue samples used to develop the CMS, PDXs are devoid of stromal cells which have important clinical and biological implications. The 5 CRC intrinsic subtypes (CRIS) have distinct molecular, functional, and phenotypic characteristics: (1) CRIS-A: mucinous, glycolytic, enriched for microsatellite instability or KRAS mutations; (2) CRIS-B: TGF- β pathway activity, epithelial–mesenchymal transition, poor prognosis; (3) CRIS-C: elevated EGFR signalling, sensitivity to EGFR inhibitors; (4) CRIS-D: WNT activation, IGF2 gene overexpression and amplification; and (5) CRIS-E: Paneth cell-like phenotype, TP53 mutations. CRIS classes could be clustered into two major subfamilies: (1) CRIS-A and CRIS-B, and (2) CRIS-C, CRIS-D, and CRIS-E. CRIS-A and CRIS-B tumours were more likely to be right-sided lesions exhibiting MSI,

mucinous histology, and CIMP. Prognostic associations between CRIS classes were also observed with a trend toward better prognosis for CRIS-D tumours and worse prognosis for CRIS-B tumours independent of clinical stage. Furthermore, CRIS-C tumours were significantly over-represented among cetuximab-sensitive tumours and depleted from resistant cases.

The association between molecular characteristics and survival has also been evaluated. Phipps *et al.* classified 2500 patients into 5 subtypes based on microsatellite instability, CIMP, BRAF and KRAS status: Type 1; MSI-high, CIMP positive, BRAF positive, KRAS negative; Type 2; MSS or MSI-low, CIMP positive, BRAF positive, KRAS negative; Type 3; MSS or MSI low, non-CIMP, BRAF negative, KRAS positive; Type 4; MSS or MSI-low, non-CIMP, BRAF negative, KRAS negative; and Type 5; MSI-high, non-CIMP, BRAF negative and KRAS negative³². Type 4 was the most common subtype, while patients with type 2 tumours had the highest disease-specific mortality, and patients with type 5 tumours had the lowest disease-specific mortality. These data show that identification of molecularly homogeneous subgroups is essential to predict prognosis, aid risk stratification, and guide clinical decision making.

1.1.3 Clinical Management of colorectal cancer

Diagnosis and staging

Diagnosis of colorectal cancer is based on lower gastrointestinal endoscopy and biopsy, confirming histological evidence of malignancy. Rectal cancer is defined as adenocarcinoma with 15cm of the anal verge on rigid sigmoidoscopy. Clinical staging is based on the 8th Edition of the American Joint Committee on Cancer (AJCC) Tumour, Node, Metastasis (TNM) system (detailed below), and involves a combination of radiological imaging techniques depending on the anatomical location of the tumour. Computed tomography (CT) of the thorax, abdomen and pelvis is performed on all patients to assess for metastatic disease. In the case of rectal tumours, pelvic magnetic resonance imaging (MRI), endoluminal ultrasound (US) and/or examination under anaesthesia are also performed for the purposes of nodal staging, and for pre-operative and operative planning. Once histological diagnosis is confirmed and radiological staging is complete, clinical management is discussed at a multidisciplinary meeting.

Primary Tumour (T)

1. TX – Primary tumour cannot be assessed
2. T0 – No evidence of primary tumour
3. Tis – Carcinoma in situ: intraepithelial or invasion of lamina propria
4. T1 – Tumour invades submucosa
5. T2 – Tumour invades muscularis propria

6. T3 – Tumour invades through the muscularis propria into the pericolic or perirectal tissues
7. T4a - Tumour penetrates to the surface of the visceral peritoneum
8. T4b – Tumour directly invades or is adherent to other organs or structures.

Regional Lymph Nodes (N)

1. NX – Regional lymph nodes cannot be assessed
2. N0 - No regional lymph node metastasis
3. N1 – Metastasis in 1-3 regional lymph nodes
4. N1a – Metastasis in one regional lymph node
5. N1b – Metastasis in 2-3 regional lymph nodes
6. N1c – Tumour deposit(s) in the subserosa, mesentery, or non-peritonealised pericolic or perirectal tissues without regional nodal metastasis
7. N2 – Metastasis in 4 or more regional lymph nodes
8. N2a- Metastasis in 4-6 regional lymph nodes
9. N2b – Metastasis in 7 or more regional lymph nodes

Distant Metastasis (M)

1. M0 – No distant metastasis
2. M1 – Distant metastasis
3. M1a – Metastasis confined to one organ or site (for example, liver, lung, ovary, nonregional node(s)) without peritoneal metastases
4. M1b – Metastases in more than one organ/site or the peritoneum

5. M1c – Metastasis to the peritoneum with or without other organ involvement

Table 1. AJCC staging of colorectal cancer 8th edition

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

Note: cTNM refers to clinical stage. pTNM refers to pathological stage (i.e. based on analysis of the resected surgical specimen). The y prefix denotes neoadjuvant therapy. ypT0N0M0 indicates a complete pathological response.

Surgery

The principle aim of oncological surgery is to optimise disease-specific outcomes without compromising recovery and quality of life.

Optimal outcome requires complete local excision of the tumour, associated draining lymph nodes and central vascular pedicle.

Resection of the mesentery along with adequate length of bowel on either side of the tumour is performed to minimise the chance of residual local disease. This is termed complete mesocolic excision in colon cancer, and total mesorectal excision in rectal cancer^{33,34}.

Dissection of the embryologically defined mesocolic/mesorectal planes to create an intact envelope of the mesocolic/mesorectal fascia, maximizes the harvesting of draining lymph nodes within the resected specimen. The operative procedure performed depends on the anatomical location of the tumour. For colonic cancer, surgery involves either a right hemicolectomy, extended right hemicolectomy or left hemicolectomy. The procedure of choice depends on the blood supply and the distribution of regional lymph nodes of the segment of colon where the tumour is located. At least 5cm of colon on either side of the tumour should be resected.

Early rectal cancers with favourable pathology (pT1, < 3 cm in diameter, well differentiated, no lymphovascular invasion) may be managed by local excision in the form of Transanal Endoscopic Microsurgery (TEMs), and the more recently adopted technique termed Transanal Minimally Invasive Surgery (TAMIS). If histopathological analysis of the resected specimen demonstrates

greater depth of invasion than pT1 or adverse histological features, completion surgery with total mesorectal excision may be performed with comparable oncological outcomes to upfront radical en bloc resection³⁵.

For more advanced rectal cancer, a high anterior resection is performed for tumours in the upper third of the rectum, whilst a low anterior resection with total mesorectal excision is performed for tumours in the middle or lower third of the rectum. An anterior resection involves removal of the rectum, distal sigmoid, mesorectum and lymphovascular pedicle of the inferior mesenteric artery. The remaining colon is anastomosed to the distal rectum or anus. A temporary defunctioning loop ileostomy may be fashioned depending on patient and operative factors. An abdominoperineal resection (APR) is performed for tumours involving the anorectal sphincter complex or for very low tumours in which an adequate distal margin would not be achieved without disrupting the sphincter. An APR involves removing the anal sphincters, rectum, distal sigmoid, mesorectum and lymphovascular pedicle of the inferior mesenteric artery. The perineal defect is closed primarily or in conjunction with a mesh or pedicle flap, and an end colostomy is formed in the left iliac fossa. Regardless of the approach, clear distal and circumferential resection margins, along with preservation of pelvic autonomic nerves, are required to optimise oncologic outcome and post-operative quality of life.

Lymph node status is the most important predictor of outcome in localised rectal cancer. The management of rectal cancer pelvic sidewall lymphadenopathy is controversial and non-standardized, with significant geographical differences. Whilst nCRT followed by total mesorectal excision is standard of care in the West, total mesorectal excision and lateral pelvic node dissection (LPND) is the primary approach in the East, particularly in Japan³⁶. The different approaches to management are reflective of differences in disease staging. Lateral pelvic lymph node involvement is considered locoregional disease in the East, and metastatic disease in the West³⁷. Randomised data to guide management are lacking, and the potential oncological benefit must be weighed against the risk of technically challenging surgery. Observational data suggest nCRT alone does not guarantee sterilisation of all lateral pelvic nodes, and that highly selective therapeutic LPND should be considered in cases of suspicious lateral pelvic nodes³⁸.

Approximately 6-10% of patients present with advanced rectal cancer involving other structures such as the bladder, uterus, vagina, prostate/seminal vesicles or sacrum³⁹. Historically, invasion of the tumour into other organs was a contraindication to surgery resulting in a median survival of <1 year⁴⁰. Palliative chemotherapy was considered the only option. Increasingly, radical pelvic exenterative surgery is being performed as it remains the only potentially curative treatment modality. The most important predictor of survival is achievement of a negative resection margin (R0)⁴¹. While neoadjuvant

chemotherapy may improve survival, it does so at the increased risk of perioperative morbidity⁴¹.

Operative approaches for CRC have evolved over the past few decades. Historically performed by an open approach, the modern era has seen the development of minimally invasive techniques including laparoscopic and robotic surgery. Advantages of these approaches include enhanced post-operative recovery, reduced opiate analgesic requirements, quicker recovery of bowel function and shorter length of hospital stay. Robotic surgery eliminates many of the technical challenges faced in laparoscopic surgery by improving visualization, manoeuvrability of instruments and ergonomics. Comparable oncological outcomes have been demonstrated with all three approaches, and so choice depends on surgeon preference and patient suitability⁴²⁻⁴⁵. Anatomical constraints of the pelvis and technical limitations of laparoscopic technology have led to the development of transanal approaches. Transanal dissection of the distal rectum (taTME) has emerged as a new technique to overcome the poor visualisation and limited access to the narrow pelvis encountered at laparoscopy, particularly for bulky mid-rectal or very low tumours in an obese male patient. Although theoretically promising, high local recurrence rates were observed in several countries following its implementation⁴⁶. These data have led to a pause on uptake of taTME until further clarification. The introduction of any new surgical technique must include clear technical standardisation, diligent training, and rigorous monitoring of outcomes.

Multimodality therapy

Although complete surgical excision is central to curative treatment of CRC, improved oncological outcomes have been achieved by the addition of systemic neoadjuvant and adjuvant therapy⁴⁷⁻⁵⁰. Combined modality treatment strategies for colon and rectal cancer differ on the basis of the natural history of the disease, in particular recurrence patterns.

Colon cancer

Upfront surgery is the standard of care for non-metastatic colon cancer. Distant disease failure however occurs in up to 50% of patients with localized disease who undergo surgery with curative intent⁵¹. Accordingly, adjuvant chemotherapy is administered in a bid to eradicate occult micrometastases and improve disease control. Early trials demonstrated superior oncological outcomes with 6 months of fluoropyrimidine-based treatment⁵²⁻⁵⁴. Subsequently, three phase 3 clinical trials showed that the addition of oxaliplatin improved disease-free survival^{49,50,55-57}. As a result, a 6-month regimen of FOLFOX (fluorouracil, leucovorin, and oxaliplatin) or a 3-month regimen of CAPOX (capecitabine and oxaliplatin) became the standard adjuvant therapy in stage III disease⁵⁸. Administration of adjuvant chemotherapy for stage II disease is on the basis of pathological risk factors. Current guidelines recommend that patients with intermediate risk disease (MSS tumours with at least one of the following: lymphatic, perineural, or vascular invasion, obstructing tumour, or pre-operative CEA >5ng/ml) receive 6-months of de

Gramont regimen (fluorouracil and leucovorin) or capecitabine⁵⁹. Patients with high risk disease (pT4, <12 lymph nodes resected, or multiple intermediate risk factors, regardless of MMR status) should receive 6-months of FOLFOX or 3-months of CAPOX⁵⁹. Neoadjuvant therapy is not routinely administered for colon cancer. Preliminary data from the FOxTROT trial which evaluated neoadjuvant chemotherapy among patients with clinical stage II and III disease found that pre-operative FOLFOX was associated with marked pathological downstaging with some patients achieving a complete pathological response (pCR), and a trend toward better disease control at 2 years.

Rectal cancer

Combined-modality therapy was a paradigm shift in managing locally advanced rectal cancer (LARC) in the latter part of the 20th century. High local recurrence rates with surgery alone prompted the development of a multimodal approach. Neoadjuvant chemoradiotherapy (nCRT; long-course radiotherapy (45-50.4Gy) with concomitant fluoropyrimidine-based chemotherapy over 6 weeks) followed by interval total mesorectal excision is now the standard of care for patients with aggressive stage II disease (bulky cT3/4 tumours, extramural venous invasion, threatened mesorectal margin) or predicted node positive disease (stage III). Several series and meta-analyses have demonstrated superior oncological outcomes compared to surgery alone^{47,48,60}. Combined-modality therapy facilitates tumour downstaging, improves resectability and reduces

local recurrence. Five-year local recurrence rates have decreased to 5% or less following the widespread adoption of neoadjuvant CRT and interval total mesorectal excision⁶¹. Short-course radiotherapy (SCRT) consisting of 25Gy delivered in 5 fractions during a one-week period (without radiosensitising chemotherapy) followed by immediate surgery is an alternative option. Patients with early stage disease (T1-2 N0) may proceed directly to surgery following diagnosis.

Neoadjuvant therapy has the potential to eradicate tumours entirely. A complete pathological response (pCR), defined as an absence of tumour cells in the resected specimen, represents a strong positive prognostic indicator, associated with excellent local disease control and disease-specific survival. In a meta-analysis of 16 studies involving 3,363 patients with LARC treated with nCRT, those who achieved a pCR had less local recurrence (OR 0.25 95% CI 0.10 – 0.59, $p = 0.002$) and better 5-year disease-free survival (OR 4.33 95% CI 2.31 – 8.09, $p < 0.001$)⁶². The challenge is to identify patients at the time of diagnosis likely to derive a meaningful benefit from multimodal therapy. Select patients in whom neoadjuvant CRT eradicates the disease may be suitable for an organ-preserving approach, avoiding the morbidity of surgery and a permanent colostomy. Conversely, identifying those patients who are unlikely to respond would avoid the morbidity and toxicity of futile CRT, and delay to definitive surgery. Furthermore, the accurate identification of a pCR represents a considerable limiting factor. The only reliable method of determining pathological response is an oncological

resection, and histological assessment of the resected specimen.

Reliable predictive biomarkers and advanced radiological imaging are required to aid pre-operative identification of a pCR.

Traditionally, adjuvant chemotherapy has been recommended in the management of LARC. This is largely based on the results of trials in colonic cancer demonstrating improved disease-free and overall survival^{49,50}. Early trials in rectal cancer also observed similar survival advantages, however these trials were conducted prior to the introduction of neoadjuvant therapy. The role of adjuvant chemotherapy in the modern era of nCRT is less defined. Four randomised European trials have failed to demonstrate a significant survival benefit⁶³⁻⁶⁶. Notably, these trials demonstrated that compliance with adjuvant chemotherapy is poor following rectal cancer surgery. In the largest of these studies, the European Organisation for Research and Treatment of Cancer (EORTC) 22921 trial, over half of patients did not complete the intended four cycles⁶⁶. Any apparent absence of survival advantage with adjuvant chemotherapy may be related, in part, to poor compliance with the treatment.

Alternative approaches are required to optimise delivery and disease control. Total neoadjuvant therapy (TNT) has emerged as an attractive strategy - systemic chemotherapy given before nCRT (i.e. induction chemotherapy) or after it (consolidation chemotherapy) in the

preoperative phase. Favourable short-term outcomes include improved chemotherapy compliance and superior pathological response^{67,68}. As radiation-induced tumour necrosis is time dependent, pathological response is influenced by interval to surgery. Several large series and meta-analyses have demonstrated increased pCR rates with intervals of > 6-8 weeks following completion of nCRT⁶⁹⁻⁷¹. No significant difference in pCR rates was found after an interval of 7 weeks compared to 11 weeks between nCRT and surgery in the GRECCAR6 phase III multicentre randomised control trial. As a result, standard practice is now an interval of 8-10 weeks⁷². Whether improved pathological response observed with TNT is due to the direct effect of chemotherapy or prolonged interval to surgery or both is unclear. Long-term survival data with TNT however is limited and interpretation hampered by marked heterogeneity among neoadjuvant/adjuvant treatment regimens and interval to surgery⁶⁷.

In addition to survivorship, a potential advantage of improved tumour regression and downstaging with systemic therapy is the selective practice of non-operative management and avoidance of a stoma or challenging anastomosis. Preservation of bowel function and health-related quality of life (HR-QL) continue to represent significant challenges in the management of LARC. Low Anterior Resection Syndrome occurs in 60-90% of patients following low or ultralow sphincter-sparing surgery for rectal cancer⁷³, and is associated with a significant and sustained reduction in HR-QL^{74,75}. If long-term disease outcomes were comparable, systemic therapy with curative intent may

be an alternative to surgery in select cases. Patients who achieve a clinical complete response (no evidence of residual disease on endoscopy or radiological imaging) may be eligible for organ preservation or less radical sphincter-saving resection with salvage surgery reserved for cases of locoregional recurrence.

Predicting response to neoadjuvant/adjuvant therapy

Although, histopathological assessment (e.g. tumour morphology, growth pattern and architecture) provides valuable prognostic information allowing broad risk stratification, it has no predictive value and does not identify specific patient subgroups that respond best to conventional chemotherapy. Importantly, approximately 50% of patients who receive oxaliplatin experience significant side effects such as chronic peripheral neuropathy. As no clinically validated tests exist to predict response, a proportion of patients suffer considerable morbidity without deriving significant oncological benefit. Accurate predictive tools are required to allow precise patient selection. With this in mind, several pathological and molecular features have been evaluated as potential predictive markers of response including MMR status, tumour budding and mucinous histology.

Dichotomisation of CRC into MMR-deficient (dMMR) and MMR-proficient (pMMR) tumours by immunohistochemistry is routinely recommended for all patients with CRC. The implications of MMR status on pathological response to chemotherapy and radiotherapy are poorly understood. Available data is conflicting, limited by

heterogeneity in testing methodology and low study numbers. Several studies have reported relative resistance of dMMR colon tumours to 5-fluorouracil-based chemotherapy^{76,77}. There is also evidence to suggest that MMR proteins play a direct and indirect role in the DNA damage response induced by ionising radiation, conferring greater sensitivity^{78,79}. This MMR status-related radiosensitivity appears to depend on the dose administered. Short-course high dose radiotherapy is associated with sensitivity in dMMR tumours, while low-dose is associated with sensitivity in pMMR tumours.

Tumour budding, present in 20-40% of CRCs, is a histopathological feature of epithelial cancers whereby isolated tumour cells or clusters of fewer than 5 cells detach from the invasive margin and migrate into the surrounding stroma⁸⁰⁻⁸². Morphologically, it is considered a manifestation of epithelial-mesenchymal transition⁸³. A meta-analysis including 7821 patients found tumour budding was a negative prognostic indicator, associated with an increased likelihood of lymph node positivity, disease recurrence and disease-related mortality at 5-years⁸⁴. Several studies evaluating the prognostic value of tumour budding in rectal cancer before neoadjuvant radio(chemotherapy) found that budding was strongly predictive of a poor pathological response⁸⁵⁻⁸⁸. The relative radioresistance observed in patients exhibiting tumour budding may be related to the combination of tumour cell 'stemness' and a poor immune response, as tumour budding has been infrequently encountered in patients with high peritumoral CD8+ T cell infiltration⁸⁹.

Mucinous tumours are defined as lesions in which >50% of the tumour volume consists of pools of extracellular mucin and account for approximately 5-15% of CRCs^{90,91}. Representative of a negative prognosis, mucinous tumours are associated with poorer response to nCRT, higher positive margins and worse survival compared to non-mucinous tumours^{92,93}. Reduced responsiveness to radiotherapy and 5-FU based chemotherapeutic regimes has been observed in mucinous tumours⁹⁴⁻⁹⁶. Poor response may be related to less apoptosis as apoptosis is a known mechanism by which radiotherapy exerts its anti-tumour effect. It is also possible alternate oncogenic pathways play a role. Mucinous histology is positively associated with MSI, BRAF mutations and CIMP-high status, and negatively associated with altered p53 expression⁹⁷.

Modern oncotherapeutic strategies

Despite advances in combined modality therapy, up to 20% of patients with rectal cancer will exhibit no or minimal response to neoadjuvant therapy⁹⁸. Furthermore, distant disease failure rates for both colon and rectal cancer remain disappointingly high, ranging between 20-30%, and account for most cancer-specific mortality⁴⁷. Recently, enhanced understanding of the dynamic and complex tumour microenvironment (TME) enabled development of novel molecularly-directed, individualised treatment. The identification of prognostic and predictive biomarkers has resulted in risk-adjusted stratification systems and therapeutic algorithms based on components of the TME. Increasing emphasis has been placed on the role of the immune

response in tumorigenesis, therapy, and predicting prognosis.

Modifying immune system anti-tumour responses are promising oncotherapeutic strategies with some strikingly encouraging results observed in a range of malignancies including CRC.

1.2 Early age onset colorectal cancer

1.2.1 Definition

There is currently no standardised and internationally accepted definition of EOCRC. Age less than 50 years at diagnosis is generally considered early onset, as most national screening programmes commence at this age. Studies reporting on the subject use varying cut-off ages, and significant differences have been observed even among patients aged under 50 years, suggesting a role for further age-based subgrouping⁹⁹.

Epidemiology

CRC rates have increased dramatically among 20-49 year olds over the last 25 years^{8,100}. The most notable change involves cancers of the distal colon and rectum. Rectal cancer rates in Europe have increased by 1.8% per year from 1990-2016⁸. Similar trends have been observed in the United States, Australia and Asia^{9,101}. EOCRC now accounts for 1 in 10 CRC diagnoses, representing the second most common cancer and the third leading cause of cancer-related death in this age group¹⁰². Based on current trends, the incidence rates of colon and rectal cancer are expected to increase by 90% and 124% respectively, among adults aged 20-34 years, and by 27% and 46%, respectively, for those aged 35-49 years within the next decade⁹. By 2030, 10% of colon cancers and 25% of rectal cancers will be diagnosed in individuals aged less than 50 years of age.

1.2.2 Clinical Features

Several clinical features of EOCRC differ to those of late-onset disease. Young patients typically develop tumours in the distal colon and rectum¹⁰³⁻¹⁰⁵. Importantly, the anatomical location of EOCRC may provide insights into the underlying aetiology, and disease process as there is increasing evidence that right-sided colon cancers differ biologically to left-sided colon and rectal cancers. Right-sided tumours are associated with older age, advanced stage and female sex^{106,107}. They often present with iron deficiency anaemia, whilst left-sided tumours tend to present with obstructive symptoms^{108,109}. Lymph node involvement and lymphovascular invasion are more common in right-sided tumours, with worse oncologic outcomes observed^{107,108}.

Synchronous and metachronous lesions, and advanced disease stage at presentation are more common in young patients with EOCRC compared to their older counterparts^{103,110}. Between 54-61.8% present with stage III and IV disease¹¹¹⁻¹¹⁴. Whilst screening may account for earlier disease stage at diagnosis in older patients, both patient and physician-related factors delaying evaluation of symptoms contributing to the later stage at diagnosis observed in the young. Low suspicion of cancer and symptoms which overlap with those of common benign diagnoses, may contribute to delayed evaluation. Physician-related delay in diagnosis has been reported to range between 15-50%¹¹⁵. Notably the vast majority of patients are symptomatic at diagnosis, in particular patients with rectal cancer¹¹⁶.

In a study of 1514 patients with rectal cancer, the median time from symptom onset to treatment was 217 days in those aged less than 50 years compared to 29.5 days for those aged over 50¹¹⁷. These data highlight the importance of considering CRC as a potential diagnosis in adults younger than 50 years (regardless of family history). Given that the majority of patients are symptomatic and have sporadic disease, emphasis should also be placed on education and not only on screening strategies. Educational initiatives to raise awareness among young adults, primary care physicians and clinicians are imperative to ensure timely diagnosis and intervention.

Pathological Features

Young patients with EOCRC frequently display adverse histopathological features¹¹⁸. Poorly differentiated or anaplastic disease, mucinous and signet-ring tumours, all associated with worse oncological outcomes, are also more common in patients with early onset disease than their older counterparts¹¹². The mucinous subtype of CRC represents a negative prognostic indicator, associated with poorer response to neoadjuvant CRT, higher positive margins and worse survival compared with non-mucinous tumours^{93,94}.

1.2.3 Molecular Profile

Whether EOCRC is characterised by different biomolecular processes to late onset disease is unclear. No unique targetable profile has been discovered as yet. Most key oncogenic pathways are implicated in both early-onset and late-onset disease, however there appear to be

some differences. Overall, young patients more frequently exhibit LINE-1 hypomethylation and *TP53* mutations¹¹⁹. They are less likely to harbour *KRAS*, *BRAF* V600E and *APC* mutations, or display promoter methylation of CpG islands^{103,118,120,121}. (Summarised in Table 2)

Reflex assessment of MSI status is now recommended routinely for all patients as per the National Comprehensive Cancer Network (NCCN) guidelines¹²². Subsequent genetic screening for Lynch Syndrome (LS) (following appropriate counselling and consent) should be performed in patients with loss of mismatch repair (MMR) proteins *MLH1*, *MSH2*, *MSH6*, and *PMS2*¹²³. The majority of patients with EOCRC have MSS tumours. Interestingly, early and late-onset MSS tumours display some differences. Gene expression analysis identified *CTNNB1* as one of the most over-expressed genes in MSS-young patients compared to MSS-old patients¹²⁴.

Key pathways such as Wnt/beta catenin, MAP Kinase, growth factor signalling (EGFR, HGF, PDGF) and the TNFR1 pathway have also been implicated in sporadic EOCRC¹²⁴. Upregulation of these pathways which play a critical role in cellular adhesion/motility, apoptosis, and inflammation, may in part influence the metastatic potential and chemoradiosensitivity of early-onset disease. To that end, subclassification of EOCRC according to genomic signature has been proposed¹²⁵.

CRC has been classified into four consensus molecular subtypes (CMS) on the basis of distinguishing molecular characteristics³⁰. In a retrospective analysis of patients with CRC aged less than 40 years, CMS1 (MSI with immune infiltration and activation) was the most common subtype (46%), while CMS3 (‘*metabolic*’ with metabolic dysregulation and KRAS mutations) and CMS4 (‘*mesenchymal*’ with marked stromal infiltration, TGFβ activation and angiogenesis) were uncommon (4% and 13%, respectively $p=0.003$)¹⁰³. CMS2 (‘*canonical*’ with WNT and MYC activation) was similar across age groups. Although CMS1 was the most prevalent subtype, the majority of patients with EOCRC have sporadic MSS tumours. The role of the immune system in early-onset disease remains largely undefined.

Aetiology

Unravelling the exposome of EOCRC is critical to understanding what drives the disease. Several risk factors have been suggested such as a westernised diet, obesity, alcohol, smoking, antibiotics, infections and alterations to the gut microbiome. A high fat diet generates procarcinogenic advanced glycation end-products, whilst visceral obesity is known to have proinflammatory effects as well as promote dysbiosis^{11,126}. Women with obesity have a nearly doubled risk of EOCRC compared to those with normal BMI¹²⁷.

Childhood/adolescent obesity has been linked to colon cancer but not rectal cancer¹²⁸. Antibiotics in childhood have been postulated to alter the microbiome toward an oncogenic phenotype however, data are lacking.

Table 2. Pathological features and molecular profile of EOCRC

Pathological features

Poor differentiation

Mucinous

Signet-ring morphology

Perineural/venous invasion

Molecular profile

Microsatellite instability

More likely to exhibit LINE-1 hypomethylation and *TP53* mutations

Less frequently harbour *KRAS*, *BRAF* V600E and *APC* mutations

Promoter methylation of CpG islands

1.2.4 Hereditary EOCRC

A hallmark of inherited cancer predisposition is young age at diagnosis. The estimated prevalence of hereditary cancer syndromes in patients with EOCRC ranges between 5-35%, compared to 2-5% of colorectal cancers overall^{118,129-131}. Lynch syndrome (LS), the most commonly diagnosed hereditary cancer syndrome implicated in the pathogenesis of EOCRC, is due to defects in the DNA mismatch repair proteins (*MLH2*, *MLH6*, *MSH2*, *PMS2*)^{131,132}. The lifetime risk of developing CRC in LS is between 50-70% and in 40% the onset of CRC is before the age of 40¹³³. Approximately one-third of EOCRC in patients less than 35 years is associated with LS^{131,134}.

Surgical approach in LS is based on the risk of metachronous CRC,

which depends on the variant a carrier has and the management of the primary cancer. The European guidelines from the European Hereditary Tumour Group and European Society of Coloproctology recommend standard segmental resection for a first colonic cancer in *MSH6* or *PMS2* pathogenic variant carriers, while extended surgery (subtotal colectomy and ileosigmoidal anastomosis or total colectomy and ileorectal anastomosis) is preferable in those who are *MLH1* or *MSH2* pathogenic variant carriers¹²³. Extended surgery is recommended for a metachronous colonic cancer with previous segmental colectomy, regardless of pathogenic variant¹²³. For a first rectal cancer, standard resection (anterior resection or abdominoperineal resection) is advised for all variants¹²³. In the case of a synchronous colonic cancer, extended surgery can be considered. All surgical decision making should be individualized, taking into account age, gender, predicted functional outcome, quality of life and other personal priorities (e.g. fertility).

The development of multigene panel testing has expanded the spectrum of known germline mutations predisposing to EOCRC. *SMAD4*, Checkpoint Kinase 2 (*CHEK2*) and *POLE*, as well as gene alterations of uncertain clinical relevance (variations of unknown significance) have all been implicated. Multiple genes associated with various hereditary cancer syndromes can now be identified with next-generation sequencing. Importantly, a significant proportion of patients diagnosed with EOCRC who do not report a family history of CRC, harbour a gene alteration associated with inherited cancer

predisposition¹³⁴. This group of patients at high risk of developing EOCRC would not meet the current criteria for early screening.

1.2.5 Oncological Outcomes

Survival data for EOCRC is limited and conflicting. Several studies report a worse prognosis, whilst others demonstrate equivalent or superior outcomes in young patients, despite presenting with more advanced disease stage and adverse histopathological features^{112,113,135,136}. Early age of disease onset is not considered in current therapeutic algorithms for localized or metastatic CRC, although patients with EOCRC are more likely to receive neoadjuvant chemoradiotherapy and adjuvant chemotherapy^{114,135}.

Why increased access to treatment may not translate into improved disease-specific outcomes is unclear. Due to the historically small proportion of patients under 50, the sensitivity of EOCRC to standard chemo(radio)therapy is not known in isolation. Conventional chemotherapeutic agents for example appear to confer minimal survival gain in the adjuvant setting¹¹⁴. Patients with EOCRC are more likely to receive neoadjuvant and adjuvant therapy outside of current guidelines (stage I and II) but experienced only minimal gain in adjusted survival compared with their older counterparts who received less treatment^{113,114,135,136}. A nationwide North American study found that adjuvant chemotherapy was administered to 50.5% of young patients vs 19.1% of older patients with low risk stage II disease¹¹⁴. Furthermore, young patients were more likely to receive multi-agent regimens rather than single agent therapy.

A focus of modern oncotherapeutic strategies has been on modifying immune system anti-tumour responses. Promising results with checkpoint blockade has been observed in MSI CRC¹³⁷. Patients aged less than 50 years however, accounted for only a small percentage of the overall study population. It could be postulated that as immune function declines with age¹³⁸, a more robust peritumoral immune response may occur in individuals with EOCRC compared with their older counterparts, potentially resulting in increased sensitivity to immunotherapy.

EOCRC poses many challenges. The underlying molecular profile and key drivers of disease remain incompletely understood. The potential of the immune system as a therapeutic target is unknown. Treatment algorithms tailored to the biomolecular signature of the tumour are needed to optimise disease control, avoid the morbidity of futile treatments, and enhance quality of life and survivorship. Data on the subject are lacking and predominantly retrospective. The aetiology, molecular typing, and genetic profile of needs to be deciphered in order to standardise age-specific preventative and therapeutic strategies.

1.3 Cancer and the immune response

1.3.1 Immunoediting

The ability of the immune system to paradoxically constrain and promote tumour development and progression was first described by Sir Frank MacFarlane Burnet in 1957¹³⁹. This process, referred to as cancer immunoediting, consists of 3 phases: elimination, equilibrium, and escape¹⁴⁰. During the elimination phase synergism between the innate and adaptive immune systems facilitates recognition and destruction of tumour cells that have escaped cell-intrinsic mechanisms of suppression, and eliminate them before tumours become clinically detectable. Tumour cells that survive the elimination phase progress to the equilibrium phase where net tumour growth is limited. They coexist with the immune system and are kept functionally dormant. Over time, persistent pressure exerted by the immune system causes the tumour cells to undergo genetic and epigenetic changes leading to the development of immune-resistant variants and progression to the escape phase. The escape phase, considered as one of the ‘Hallmarks of Cancer’¹⁴¹, is characterised by tumour cell proliferation and metastasis. Growth is unrestrained and tumours become clinically detectable. Several mechanisms, including production of cytokines TGF β and IL-10, and deactivation of the cytotoxic T cell response, enable the tumour cells to evade recognition by the immune system¹⁴².

1.3.2 The immune microenvironment

The immune microenvironment of cancer is complex, and

incompletely understood. Just as each organ is immunologically distinct, so too are the tumours that develop in them. The tumour microenvironment (TME) is comprised of diverse populations of immune cells. The type, location and density of immune cells present in the immune TME is an important predictor of survival and response to treatment¹⁴³. The diversity of tumoral immune infiltration has led increasingly to the nomenclature '*hot*' or '*cold*' tumours. '*Hot*' tumours characterised by high cytotoxic T-cell infiltration within the tumour or surrounding stroma. '*Cold*' tumours demonstrate an absence of or poor T-cell infiltration within the tumour or surrounding TME, and also include 'immune cell excluded' tumours in which T cells are present at the margin but not within the tumour¹⁴⁴. The Immunoscore is a scoring system developed to measure intra-tumoral immune response. It is based on the quantification of cytotoxic and memory T cells (CD3/CD45RO, CD3/CD8, or CD8/CD45RO) in the core of the tumour and its invasive margin¹⁴³. It provides a score ranging from 0 (low densities of both lymphocyte populations in both regions) to 4 (high densities in both regions). The Immunoscore has been internationally validated as an independent predictor of disease-specific survival in CRC, superior to the classical TNM staging system¹⁴⁵.

Cells of the immune TME

Tumour-infiltrating lymphocytes (TILs) are a major component of the TME. Throughout the immunoediting process, different T cell populations infiltrate the tumour, invasive margin and draining

lymphoid organs, and play a key role in constraining tumour growth.

Tumour-specific antigens derived from neoantigens (aberrant proteins that result from somatic mutations) evoke T cell driven responses.

Immune cells of the TME include (but are not limited to) CD8+, CD4+, natural killer, mucosal associated invariant T (MAIT) cells, and $\gamma\delta$ T cells, all of which have different characteristics and functional properties.

CD8+ cytotoxic T cells

CD8+ cytotoxic T cells recognize non self-peptide epitopes displayed by MHC class I molecules expressed on antigen presenting cells¹⁴⁶.

Upon activation, CD8+ T cells utilize 2 primary mechanisms to destroy tumour cells; secretion of cytokines (IFN γ , TNFa) which have direct anti-tumour effects, and production/release of cytotoxic granules containing granzymes and perforin¹⁴⁷. Perforin forms a pore in the membrane of the target cell, allowing the granzymes to enter.

Granzymes (B and K) are serine proteases which mediate apoptosis.

CD8+ T cells are subdivided according to their state of differentiation into naïve T cells, effector T cells, and subsets of memory T cells. In the setting of cancer (including colorectal cancer), the presence of intra-tumoral CD8+ T cells is a positive prognostic indicator¹⁴⁸⁻¹⁵⁰.

CD4+ T cells

CD4+ T cells recognize peptides displayed by MHC class 2 molecules on antigen presenting cells, and play an important role in the adaptive immune response¹⁵¹. Regulatory T cells (Tregs) are a subset of CD4+

T cells¹⁵². They are characterised by the expression of the nuclear transcription factor Forkhead box (FoxP3) and are key regulators of the immune response¹⁵³. Tregs produce immunosuppressive-mediators TGF β and IL-10, and suppress activation, proliferation, and cytokine production of CD8⁺ and CD4⁺ T cells¹⁵⁴.

Natural Killer Cells

Natural Killer (NK) cells are innate immune cells, classified as group 1 Innate Lymphocytes (ILCs). They demonstrate spontaneous cytolytic activity secreting several cytokines (TNF α , IFN γ) and chemokines without prior activation¹⁵⁵. NK effector function is regulated by signals derived from activating and inhibitory receptors¹⁵⁶. Healthy cells express MHC 1 receptors which marks these cells as ‘self’^{157,158}. Inhibitory receptors recognise MHC 1 and prevent NK-mediated cytolytic activity. Tumour cells lose their MHC 1 making them a target for NK cells.

Mucosal associated invariant T cells

Mucosal associated invariant T (MAIT) cells are unconventional innate-like T cells defined by the expression of semivariant $\alpha\beta$ T cell receptors¹⁵⁹. They are abundant in peripheral blood, liver, and the gastrointestinal tract¹⁶⁰. MAIT cells are not MHC restricted and recognise the MHC related protein MR1¹⁶¹. Once activated, they produce a range of cytokines including IFN γ , TNF α , IL17, perforin, and granzyme B¹⁶²⁻¹⁶⁴.

$\gamma\delta$ T cells

$\gamma\delta$ T cells are a group of heterogeneous unconventional T cells that account for a small proportion of T cells overall. They express a unique T cell receptor composed of γ and δ chains and demonstrate tissue-specific localisation of subpopulations¹⁶⁵. Whilst $\gamma\delta$ T cells only comprise 1-5% of lymphocytes in peripheral blood, 20% of intra-epithelial lymphocytes in the colon express $\gamma\delta$ TCRs^{166,167}. They display marked functional plasticity, acting as a bridge between the innate and adaptive immune systems. Specific features of $\gamma\delta$ T cells include non-MHC restricted antigen recognition and secretion of cytokines, making them attractive targets for immunotherapy^{168,169}.

T cell states in cancer

Although the presence of TILs is a positive prognostic marker, these cells fail to eradicate the tumour. One reason for this is the curtailing of T cell effector function. Chronic antigen exposure results in T cell receptor (TCR) signaling, leading to persistent and elevated expression of inhibitory receptors such as Programmed cell death protein-1 (PD-1), T-cell immunoglobulin (Ig) mucin 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT). While transient PD-1 expression is a characteristic of normal T cell activation, sustained expression drives effector dysfunction and T cells enter an 'exhausted' or dysfunctional state which represents a distinct state of differentiation¹⁷⁰. Over expression of other inhibitory receptors follows, with co-expression increasing as dysfunction progresses. This

dysfunctional state is characterized by increased expression of inhibitory receptors, reduced proliferative capacity and effector function (production of IFN γ , TNF α , and IL-2), in addition to transcriptional and metabolic changes¹⁷¹.

Dysfunction occurs gradually along a gradient of pre-dysfunction, early dysfunction, and late dysfunction, rather than a binary differentiation of state, and is regulated by various transcription factors such as eomesodermin homolog (EOMES), T-box expressed in T cells (T-BET) and Tcf-1 (encoded by Tcf-7). Proliferative capacity and effector function is maintained during the pre-dysfunctional and early dysfunctional states, and lost in late dysfunction. Although these regulating transcription factors may distinguish different states along the continuum of dysfunction, considerable transcriptional and functional heterogeneity exists among dysfunctional T cells¹⁷².

Despite loss of effector function in late dysfunction, expression of the genes encoding these factors remains high. Thus, transcription profile may not always accurately predict effector capacity. Additionally, even in late dysfunction, CD8⁺ T cells have the capacity to secrete CXCL13, a B-cell attractant suggesting they are not inert in the exhausted state but acquire a novel function. Transcriptional and functional diversity among dysfunctional T cells gives rise to distinct subpopulations. 'Progenitor' T cells express intermediate levels of PD-1, whilst those that are terminally exhausted exhibit high levels of PD-1 and other co-inhibitory receptors¹⁷³.

Metabolic changes in T cell dysfunction

Upon TCR engagement, T cells undergo metabolic changes to meet the energy demands of proliferation and effector function. While naïve T cells rely on oxidative phosphorylation (OXPHOS), activated T cells switch to aerobic glycolysis to generate ATP. In dysfunctional T cells these metabolic pathways are altered. These alterations include reduction in utilization of glucose, and dysregulation of mitochondrial fatty acid oxidation. T cells are highly dependent on the availability of nutrients in the TME to generate energy. Activated T cells that do not upregulate their intrinsic metabolic pathways acquire a hyporesponsive phenotype rendering them unable to carry out their proliferative and effector functions. Several metabolic changes not only deplete the TME of essential nutrients required for T cell function, but also lead to the accumulation of immunosuppressive metabolites. Limitation of oxygen during tumour growth reduces energy production via OXPHOS, increasing dependency on glycolysis. Hypoxia induces increased expression of the transcription factor hypoxia-inducible factor 1 α (HIF-1 α) which promotes expression of inhibitory receptors on T cells¹⁷⁴. The availability of amino acids also impacts T cell function. Glutamine which is essential for T cell activation and differentiation is often used by tumour cells. This creates a TME with low glucose, low amino acids, and low pH, all of which impair T cell function.

Immune signatures

Various immune signatures have been identified in CRC¹⁷⁵. CRC is

associated with a wound-healing immune phenotype characterised by a high proliferative activity and intra-tumoral heterogeneity with a low T helper type 1 (pro-inflammatory) to T helper type 2 (anti-inflammatory) ratio¹⁷⁶. As the gastrointestinal tract is continuously exposed to pathogens, tight regulation of tissue-resident immune cells is required to ensure homeostasis and prevent autoimmunity.

1.3.3 Tumour biology and the immune response

Intra-tumoral immune responses are influenced by a tumour biology. Significant variations in mutational frequencies or tumour mutational burden (TMB) have been reported across cancer types¹⁷⁷. Tumours with high TMBs are more likely to express immunogenic neoantigens that can be recognized by T cells, and consequently more likely to evoke a strong immune response and respond to immunotherapy. With defective DNA mismatch repair, inability to correct mismatches in DNA replication results in the accumulation of multiple insertion and deletion mutations at coding microsatellites which in turn generates highly immunogenic frameshift peptide (FSP) neoantigens^{178,179}. The high load of neoantigens is thought to ignite the strong local immune response observed in MSI tumours. This response is characterised by dense cytotoxic T-cell infiltration associated with a Crohn's-like lymphocytic reaction and a favourable pro-inflammatory, IFN γ dominant Th1 response¹⁸⁰. In CRC, immune-enhancing therapies have been more successful in patients with high TMBs (MSI tumours) compared to those with lower TMBs (MSS tumours)¹³⁷. Nonetheless, T cell infiltration has also been observed in

tumours with low TMBs¹⁸¹, indicating that mutational profile alone does not control the immune TME or predict treatment response.

The enhanced immunogenicity of MSI tumours may in part explain their favourable prognosis compared to MSS tumours¹⁸². Despite a strong peritumoral lymphocytic reaction, the immune system is unable to eradicate the tumour. Multiple mechanisms in which MSI tumours defy immunological control have been described. One of the most common mechanisms is loss of HLA class I antigen due to mutations in the beta-2-microglobulin gene (B2M). These mutations are found in almost one third of MSI tumours but very infrequently in MSS¹⁸³. They are associated with better disease-free survival^{184,185}. Notably, the majority of patients studied had pathological stage II disease and whether this protective effect extends to stage III (node positive) disease is unknown. The exact cellular interactions underling the protective effect are unclear. Whilst the absence of HLA class 1 expression enables tumour cells to avoid T cell recognition, natural killer cells (NK) become activated¹⁸⁶. This phenomenon is termed ‘missing self’ and results in NK-mediated destruction of circulating tumour cells¹⁸⁷. Thus, B2M testing may represent a useful prognostic tool in MSI tumours.

Lynch Syndrome-associated MSI CRC (i.e. cancer occurring in the context of LS) and sporadic MSI CRC differ immunologically. As the lifetime risk of developing CRC in LS is between 50-70% (not 100%), immune surveillance protects against some degree of tumour

development¹³³. Higher T cell infiltration has been found in LS-associated MSI CRC compared with sporadic MSI CRC¹⁸⁸⁻¹⁹⁰. The presence of FSP-specific T cell reactivity in healthy patients with LS suggests presensitisation of the immune system and may account for the stronger T cell reaction observed in LS-associated CRC¹⁹¹. A small clinical trial investigating whether vaccination with MMR deficiency-induced neoantigens could provoke an adaptive immune response and prevent tumour development, has shown promising results without the occurrence of serious adverse events¹⁹².

1.3.4 Multimodal therapy and the immune response

The impact of altered immune responses following systemic chemotherapy, either in the neoadjuvant or adjuvant setting, remains poorly understood and is not considered in current treatment strategies. Chemotherapy stimulates anti-tumour immune responses, but it may also deplete functional immune cells. Depletion of all the main subtypes of circulating lymphocytes has been observed for up to 12 months following standard chemotherapy in non-colorectal cancers¹⁹³. The effect of immunosuppression at a time when the immune system should be at maximal functional capacity is unknown. Understanding the biomolecular mechanisms of treatment-related immune dysfunction may guide strategy approach (e.g. neoadjuvant vs adjuvant therapy).

Oncotherapeutic synergism

Modern oncology focused on optimising the potential synergy of conventional (chemotherapy, radiotherapy) and novel (monoclonal antibodies) treatments to increase tumour immunogenicity. An understanding of the immune effects of each therapy is essential when designing combined modality strategies (Figure 1). Cytotoxic chemotherapy is one apex of the traditional tripartite of CRC treatment, alongside radiotherapy and surgery, and exerts a multitude of immunomodulatory effects. Historically considered as immunosuppressive, cytotoxic chemotherapy activates anti-tumour immune responses by directly stimulating T cell responses, and inhibits immunosuppressive cells (Tregs and myeloid-derived suppressor cells)^{194,195}. Immunogenic cell death has been observed with several chemotherapeutic agents^{196,197}. Moreover, recent data suggests intra-tumoral immune response as measured by the Immunoscore, may predict the therapeutic benefit of adjuvant oxaliplatin-based chemotherapy in patients with stage III colon cancer¹⁸¹.

Radiotherapy (RT) remains an important component of treatment for locally advanced rectal cancer (LARC) as it reduces the risk of local recurrence when given neoadjuvantly⁴⁸. As well as exerting direct genotoxic effects on tumour cell DNA (culminating in apoptosis), RT increases innate and adaptive immune signaling pathways in the local tumour environment. It also stimulates a systemic immunogenic response through circulating chemokines and cytokines¹⁹⁸, which may

account for the regression of distant metastases outside the irradiation field, a phenomenon termed the abscopal effect¹⁹⁹.

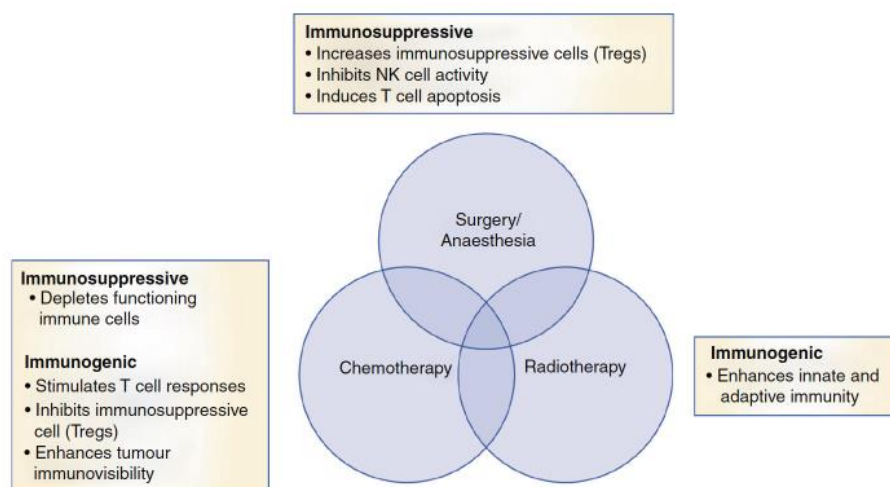


Figure 1. The immunomodulatory effects of surgery, chemotherapy, and radiotherapy. The various aspects of cancer treatment have immunogenic and immunosuppressive effects²⁰⁰.

Neoadjuvant therapy

Colonic and rectal cancers differ in terms of molecular landscape and patterns of disease recurrence^{201,202}. These differences are reflected in the different management approaches of each cancer. In general, colonic cancer is treated with upfront surgery followed by adjuvant chemotherapy for stage III disease and select cases of stage II disease. Rectal cancer requires a more complex treatment strategy consisting of neoadjuvant therapy, interval surgery and adjuvant chemotherapy. Neoadjuvant chemoradiotherapy is the standard of care for LARC (defined as bulky cT3/4 tumours or predicted node positive disease). It facilitates tumour downstaging, improves resectability and reduces local recurrence rates. Traditionally, 5 fluorouracil-based

chemotherapy has been administered alongside RT as a radiosensitising agent. The combination of chemotherapy and radiotherapy increases local immune responses evidenced by a higher density of cytotoxic TILs that may be associated with better disease-free survival²⁰³⁻²⁰⁵. Individual response to nCRT however varies, with up to 25% of patients achieving a pCR⁶². Immunological factors associated with pathological response remain incompletely understood, and involve a complex interplay between various T cell subsets and the tumour microenvironment. High pre-treatment CD8+ T cell density, high CD4+ T cell density and low Myeloid-Derived Suppressor Cell density are associated with a higher likelihood of tumour regression and achieving a pCR^{203,206,207}.

Total neoadjuvant therapy, whereby some or all of the planned chemotherapy (typically oxaliplatin) is given as either an induction or consolidation strategy, represents a promising option for LARC²⁰⁸. Two phase III trials presented showed that neoadjuvant chemotherapy is effective in this setting^{209,210}. Whilst favourable short-term outcomes include improved chemotherapy compliance and superior pathological response, the impact on long-term disease-specific outcomes remains to be defined (although preliminary results suggest a survival benefit)^{67,68,211}. Data on the immune effect of neoadjuvant chemotherapy is limited. A small pilot study found that FOLFOX was associated with increases in T cell infiltration, and MHC-I and PD-1 expression compared to pre-treatment levels, suggesting CT-mediated

priming of the tumour-immune microenvironment²¹². No correlation with pathological response was observed.

A focus of modern trials has been on increasing pathological response rates to facilitate less radical surgery or even organ preservation in select patients. In order to optimize management, an understanding of the interplay between tumour biology, the immune system, and systemic therapies is imperative.

1.4. Tumoral immunogenicity and immunotherapy

1.4.1 Intra-tumoral immune response

Intra-tumoral T-cell infiltration is not only a powerful prognostic indicator. The immunoscore is also a predictive marker of chemotherapy efficacy in patients with stage III colon cancer. A multicentre study of 763 patients found that those with an intermediate or high immunoscore benefited the most from adjuvant chemotherapy²¹³. Patients with a low immunoscore who did not receive chemotherapy had similar recurrence risk as those who received chemotherapy.

In the randomised clinical phase 3 IDEA trial, the immunoscore was also predictive of disease-free survival benefit with longer duration adjuvant mFOLFOX6¹⁸¹. Patients with an intermediate or high immunoscore significantly benefited from 6-month treatment compared to 3-month treatment. Conversely, in patients with a low immunoscore, no significant benefit of 6-month mFOLFOX was observed compared to 3-month treatment. Although these data need to be validated in an external independent cohort, they support the incorporation of the immunoscore into routine clinical practice and the development of a new TNM-Immune (TNMI) classification system.

1.4.2 Inhibitory checkpoint therapy

The discovery of the immune checkpoint, Programmed cell death protein-1 (PD-1) represents an exciting development in

oncotherapeutics. Inhibitory checkpoints are proteins expressed on the surface of activated T cells following T cell receptor (TCR) engagement with tumour antigens. T cell effector function is suppressed when these checkpoints bind to their ligands (e.g. PD-1 and PD-L1 or PD-L2). Tumour cells reduce T cell mediated anti-tumour response by upregulating these ligands²¹⁴. Chronic antigen exposure and TCR signalling results in persistent checkpoint expression on tumour infiltrating lymphocytes (TILs). An ‘exhausted’ or dysfunctional state occurs, characterised by reduced cytotoxicity¹⁷¹. Monoclonal antibodies (MAB) against checkpoint receptors can block this interaction to reinvigorate effector function and enhance anti-tumour responses. Recent data suggests that these MABs reinvigorate infiltrating PD-1+ T cells rather than tumour resident T cells²¹⁵.

(Figure 2)

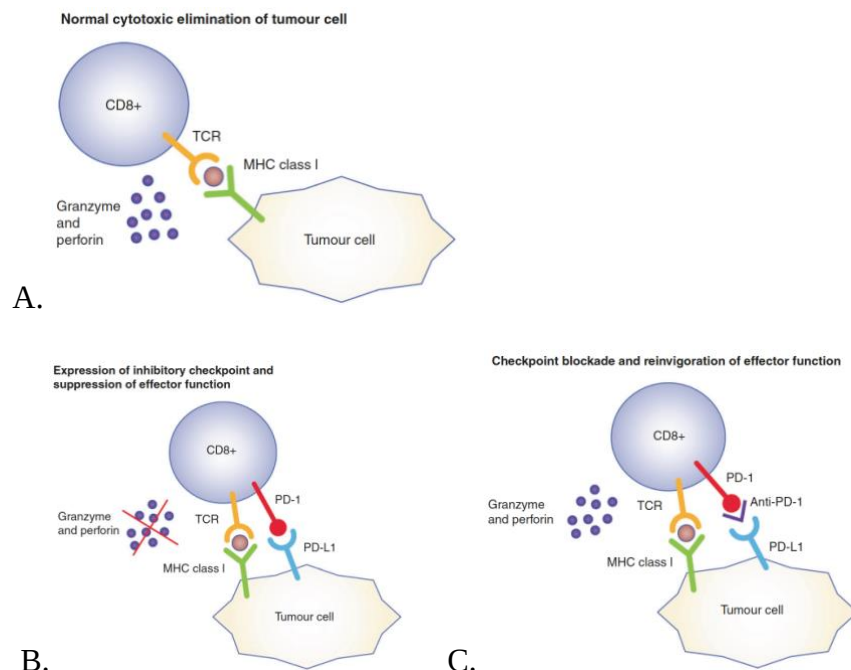


Figure 2. Activation of CD8⁺ T cells in the tumour microenvironment and mechanisms of inhibitory checkpoint blockade²⁰⁰.

1.4.3 Checkpoint therapy in colorectal cancer

The immunobiology of CRC has important therapeutic implications.

MSI tumours appear to be largely unresponsive to conventional chemotherapy. The FOxTROT phase III clinical trial evaluating neoadjuvant FOLFOX chemotherapy in locally advanced colon cancer found lower rates of tumour regression as well as disease recurrence at two years in MSI cancers compared to MSS. These data suggest MSI colon tumours should be treated differently to MSS cancers.

MMR status also appears to predict response to immunotherapy with checkpoint blockade (e.g. anti-PD-1 therapy). Significant response rates have mainly been observed in MSI tumours with limited effect seen in MSS tumours²¹⁶. In an early clinical trial of metastatic CRC, PD-1 blockade with pembrolizumab resulted in an immune-related objective response rate and immune-related progression-free survival rate of 40% and 78% in MSI tumours compared to 0% and 11% in MSS¹³⁷. Nivolumab and/or ipilimumab yielded similar results were observed in the CheckMate-142 study²¹⁷. Checkpoint therapy has more recently been evaluated in primary CRC. The NICHE study reported tumour regression in 100% of MSI tumours, with a pCR rate of 60%²¹⁸. In MSS tumours, regression was only observed in one in four patients, with CD8+ PD-1+ T cell infiltration predictive of response.

MSS tumours represent the greatest clinical challenge in CRC as their immune microenvironment remains poorly understood. Limited

response to checkpoint therapy has led to the assumption that these tumours are immunologically 'cold'. Their low mutational burden compared to MSI tumours is thought to hinder stimulation of a local immune response and enable immune evasion. It is notable however, that 1 in 5 MSS tumours have a high Immunoscore and up to 1 in 4 patients with MSS tumours demonstrate a response to checkpoint therapy^{145,218}. Checkpoint therapy trials to date have not stratified patients based on pre-treatment tumour immune infiltration CD3+ PD-1 expression. Hence, a subset of MSS tumours may be good candidates for checkpoint blockade. Combination of therapy with targeted agents (to elicit transient genomic instability) as well as checkpoint blockade may represent a promising strategy for MSS tumours. The key is to the patients with MSS tumours who display a high mutational burden and/or a robust intra-tumoral immune response, who may respond to checkpoint blockade.

1.5 Hypothesis and Aim

In this thesis, it is hypothesised that tumour biology influences the immune response in colorectal cancer. Deciphering the interplay between tumour biology and the immune system may aid the design of novel immunotherapeutic strategies and identify patients who would derive a meaningful benefit from immunotherapy. A specific focus is microsatellite instability in early age onset colorectal cancer. It is hypothesised that early age onset disease is associated with distinct immunopathological and molecular features in comparison to late onset disease, which may influence response to immunotherapy.

Specific aims

Aim 1: To evaluate the immunopathological features of microsatellite instability in early age onset colorectal cancer

Aim 2: To evaluate inhibitory checkpoint expression on tumour-infiltrating lymphocytes according to microsatellite status

Chapter 2. Materials and Methods

2.1 Patient cohort

2.2 Clinical pathway

- 2.2.1 Neoadjuvant therapy
- 2.2.2 Surgery
- 2.2.3 Perioperative protocol
- 2.2.4 Adjuvant chemotherapy
- 2.2.5 Follow up

2.3 Histopathology

2.4 Molecular profile

- 2.4.1 Mismatch repair status

2.5 Flow cytometric analysis

- 2.5.1 Materials
- 2.5.2 Study cohort
- 2.5.3 Samples collected
- 2.5.4 Sample transfer and storage
- 2.5.5 Sample processing
- 2.5.6 Data analysis

2.6 Single cell RNA sequencing

2.7 Data collection and management

2.8 Data analysis

2.1 Patient cohort

2.1.1 Institutional patient cohort

Patients were identified from a prospectively maintained database of all patients with histologically confirmed colorectal cancer treated in St Vincent's University Hospital, Dublin. Baseline demographic, clinical, staging, treatment, histopathological and survival data are recorded.

2.1.2 International patient cohort

Patients were identified from the Research in Early Age Colorectal Cancer Trends (REACCT) Collaborative database. The REACCT Collaborative is an international multicentre collaborative described in detail in section 2.7.3.

Specific eligibility criteria applied to each study described in this thesis. Each study protocol was reviewed and approved by the institutional Research and Ethics Board.

2.2 Clinical pathway

2.2.1 Neoadjuvant therapy

After histological diagnosis and radiological staging, all patients were discussed at an institutional multidisciplinary team meeting. Patients with clinical stage III or aggressive stage II rectal cancer (extramural venous invasion, T4 tumour, threatened mesorectal margin) received long-course neoadjuvant chemoradiotherapy, comprising 45–50.4Gy delivered in daily fractions over 5–6 weeks and concurrent 5-fluorouracil (5-FU)-based chemotherapy.

Following completion of neoadjuvant therapy, patients were restaged by CT of the thorax, abdomen and pelvis, MRI of the pelvis and clinical/endoscopic evaluation. If there was no evidence of local or systemic disease progression and performance status had not deteriorated significantly, total mesorectal excision was performed after an interval of 10–12 weeks.

2.2.2 Surgery

Surgery for rectal cancer involved proctectomy with total mesorectal excision (TME) by sphincter-preserving anterior resection or abdominoperineal excision of the rectum. A defunctioning loop ileostomy was routinely performed for patients undergoing an anterior resection following neoadjuvant chemoradiotherapy, or in the presence of significant comorbidities or risk factors for anastomotic leak. Surgery for colonic cancer involved colectomy depending on tumour location. The majority of cases were performed laparoscopically, with the open approach reserved for select patients

in whom a laparoscopic approach was unfeasible.

2.2.3 Perioperative protocol

Patients were managed in accordance with the Enhanced Recovery After Surgery (ERAS) protocol with early introduction of oral diet, mobilisation, and weaning of opioid-based analgesia. Serum inflammatory markers (e.g. white cell count, C-Reactive protein) were monitored regularly post-operatively. A CT scan was performed if clinically indicated.

2.2.4 Adjuvant chemotherapy

Adjuvant 5-FU based chemotherapy, either as a 5-FU infusion or oral Capecitabine, with or without the addition of oxaliplatin (FOLFOX), or irinotecan (FOLFIRI), was given routinely to patients with histologically confirmed stage III disease who were otherwise fit. For patients with stage II disease, adjuvant chemotherapy was considered in patients with unfavourable clinical or histopathological features on an individual basis after multidisciplinary discussion.

2.2.5 Follow up

All patients were followed up with annual CT and endoscopy at 1, 3 and 5 years, or when indicated clinically. Locoregional recurrence was defined as that occurring at the site of the anastomosis or within the pelvis. Distant recurrence was defined as that occurring within a solid organ. Cytological, histological or radiological proof was required to confirm a diagnosis of recurrent disease.

2.3 Histopathology

The tumour stage was defined according to the TMN staging system and the American Joint Committee on Cancer classification (AJCC).

Haematoxylin and eosin sections of the resected specimen were analysed using a minimum dataset and a standardised reporting system. The following histopathological features were analysed: glandular differentiation, mucin, tumour budding, tumour margin, lymphovascular invasion, and extramural vascular invasion.

Differentiation was classified as one of the following: (i) well differentiated (at least 95% glandular differentiation), (ii) moderately differentiated (5–95% glandular differentiation), (iii) poorly differentiated (more than 50% undifferentiated areas) or (iv) undifferentiated (less than 5% glandular differentiation). Mucinous tumours were defined as lesions in which >50% of the tumour volume consists of pools of extracellular mucin. Tumour budding was defined by the presence of isolated tumour cells and < 5 detached tumour cells at the invasive front of the tumour. Microscopically clear resection (R0) was defined as a tumour-free resection margin of at least 1 mm.

2.4 Molecular profile

2.4.1 Mismatch repair status

Microsatellite instability was assessed using immunohistochemistry for mismatch repair proteins, MLH1 (BD Bioscience, clone G168-728), PMS2 (BD Biosciences, clone A16-4), MSH2 (Calbiochem, clone FE11), and MSH6 (BD Biosciences, clone 44).

Immunohistochemistry was performed on the automated Leica BOND immunostainer. Bond ER2 solution (30 minutes) was used for antigen retrieval with antibody incubation time of 15 minutes at dilutions of 1 in 200. Visualisation of the antibody antigen reaction was via the Bond polymer detection system. Nuclear staining in any area of the tumour was classified as showing no loss of the mismatch repair proteins (MSS). Complete loss of nuclear staining of the mismatch repair proteins in the entire tumour along with positive staining of nuclei of non-neoplastic cells were classified as having loss of expression of that mismatch repair protein (MSI). Germline mutations in MMR genes (MLH1, MSH2, MSH6, PMS2) detected by genetic testing were considered Lynch Syndrome-associated MSI CRC. In patients with loss of MLH1, BRAF V600E and hypermethylation status were assessed. If both were negative, germline testing was recommended. Germline testing was recommended in all patients with loss of MSH2, MSH6, and PMS2.

2.5 Flow cytometry

Principles

Flow cytometry is a laser-based technique used for the rapid and analysis of mixed populations of cells. Applications include immunophenotyping which allows the detection and characterisation of different immune cell subpopulations within a larger heterogeneous population. Cells are stained with fluorochrome-conjugated antibodies that are targeted against antigens on the cell surface. These antigens are given “cluster of differentiation” numbers (CD numbers). Cluster of differentiation markers are surface markers that identify a particular differentiation lineage recognised by a group of monoclonal antibodies, and are used to define specific cell population. For example, T cell markers include CD3, CD8 and CD4. Populations of cells can also be defined using several surface receptors, activation states and cytokine release.

The technique of flow cytometry is based on the principle of fluorescence. A fluorescent compound has a range of specific wavelengths at which it absorbs light energy. Upon absorption of light, electrons become ‘excited’ and emit light at a longer wavelength before returning to their original ‘ground’ energy state, a process known as fluorescence. The cells are prepared in a single cell suspension and stained with fluorochrome-labelled antibodies to the specific markers of interest. The cells pass through the flow cytometer in single file where each individual cell passes by a laser light. The fluorochrome absorbs the light and re-emits it at a different

wavelength within a specific range (known as its emission spectrum).

The light emitted is detected by specific detectors within the cytometer (multiple different wavelengths can be detected at one time). Since the colour of the exciting and emitting light is different, they can be separated from one another by optical filters. Each fluorochrome has a wide emission spectrum resulting in some overlap between the fluorochromes when multiple fluorochromes are used. In order to overcome this overlap, a process named colour compensation is performed. This involves calculating how much interference a fluorochrome will have in a channel to which it is not assigned. Computer software then converts all of this data into an analysable form.

2.5.1 Materials

2.5.1.1 Flow cytometry antibodies and stains

Table 3a

Target	Colour	Clone	Catalog number	Supplier
CD45	BV785	HI30	304047	Biolegend
CD3	BV510	UCHT1	300447	Biolegend
	BV605	UCHT1	300459	Biolegend
	BV711	OKT3	317327	Biolegend
CD8	BV605	SK1	344741	Biolegend
CD4	APC	A161A1	357407	Biolegend
CD56	APC	MEM-188	304610	Biolegend
CD161	PEDazzle	HP-3G10	339939	Biolegend
TCR V δ 1	PE-Vio770	REA173	130-117-809	Miltenyi Biotec
TCR V δ 2	BV711	B6	331411	Biolegend
TCR V δ 3	APC		IM99513	Beckman Coulter
TCR V alpha7.2	BV711	3C10	351731	Biolegend
	FITC	3C10	351703	Biolegend
INF γ	PeCy7	4S.B3	502527	Biolegend
	PEDazzle	B27	506529	Biolegend
TNF alpha	AF700	Mab11	56-7349-41	Invitrogen
AREG	PE	AREG559	12-5370-42	BD Biosciences
IL17a	APCCy7	BL168	512319	Biolegend
GZMK	PerCp	GM26E7	370513	Biolegend
GZMB	BV421	QA18A28	396413	Biolegend
PD-1	BV650	EH12.2H7	329949	Biolegend
TIGIT	PerCp	A15153G	372717	Biolegend
NKp80	FITC	REA845	130-112-594	Miltenyi Biotec

Flow cytometry stains

Table 3b.

Stain	Catalog number	Supplier
Zombie Aqua™ Fixable Viability Dye	423101	Biolegend
Zombie Yellow™ Fixable Viability Dye	423103	Biolegend
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain	L10119	Invitrogen

2.5.2 Study cohort

The study cohort included patients with non-metastatic, treatment naïve, CRC undergoing surgical resection with curative intent in St. Vincent's University Hospital. Exclusion criteria included neoadjuvant chemotherapy or radiotherapy, or pre-existing immunosuppression secondary to autoimmune disorders or medication.

2.5.3 Samples collected

Samples collected included tumour and tumour-associated normal uninvolved colonic tissue ($\geq 20\text{cm}$ from tumour) and peripheral blood (collected prior to induction of general anaesthesia).

2.5.4 Sample transfer and storage

Tumour and colonic tissue were placed in sterile prepared Hanks Balanced Salt Solution (HBSS) media, placed on ice and transferred to laboratory at TBSI, Trinity College Dublin for processing (as outlined below) within 30 minutes of taking the specimen. Peripheral

blood (15mL) was collected in K₂EDTA anticoagulant tubes and stored at room temperature until transfer to the laboratory.

In the laboratory, the tumour and colonic tissue samples were weighed, dissected into 1-2mm pieces and placed in CryoStor® cell cryopreservation media. For every 0.75g of tissue, one cryovial containing 1ml of CryoStor was used. The vials were placed in a Mr Frosty for 20 minutes at 4°C and then transferred to the -80°C freezer. The Mr Frosty was pre-chilled to 4°C. Within 24 hours, the vials were transferred out of the Mr. Frosty container and placed in a container in the – 80°C freezer for long-term storage until processing.

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by density gradient centrifugation. Blood was poured into 50 ml falcons and residual was washed with HBSS. Blood was diluted with HBSS, yielding a final volume of 30 ml per tube. The blood-HBSS solution was layered slowly over lymphoprep density gradient medium and the tubes were centrifuged at 1500 rpm for 25 minutes at room temperature, brake and acceleration 0. The lymphocyte layer was removed with caution using a Pasteur pipette and washed X2 in HBSS (centrifuged at 1300 rpm for 5 minutes). The cells were re-suspended in CryoStor® cell cryopreservation media. For every 5ml of whole blood, cells were re-suspended in 1ml of CryoStor. One cryovial was used to store 1ml of PBMC-CryoStor solution. The vials were placed in a Mr Frosty for 20 minutes at 4°C and then transferred to the -80°C freezer. The Mr Frosty was pre-

chilled to 4°C. Within 24 hours, the vials were transferred out of the Mr. Frosty container and placed in a container in the – 80°C freezer for long-term storage until processing.

2.5.5 Sample processing

Sample processing involved a sequence of thawing, enzymatic digestion, cell stimulation, and live cell, extra- and intracellular staining prior to flow cytometric analysis.

Thawing tumour, colorectal tissue and PBMCs

Cryopreservation vials were removed from storage in the -80°C freezer and placed on dry ice. 20ml of sterile thawing medium (HBSS) was pipetted into 30 ml falcon tubes and warmed to 37°C. The samples were thawed by gently swirling in a 37°C water bath until the edge of the frozen cells detached from the side of the cryovial. The frozen cells were then poured into the warm thawing medium. The cryopreservation vials were washed out with thawing medium, which was then added to the cell suspension. The cells were centrifuged at $300 \times g$ for 5 minutes.

Enzymatic digestion

Tumour and colonic tissue were enzymatically digested using a standardised protocol outlined below. For every 1g of tissue, 5ml of digestion mix was required.

Digestion mix (5ml):

DNase I	50 µl
Collagenase IV	0.0048g
1% BSA	0.05g
1% FCS	50 µl
20mM HEPES	100 µl
RPMI	

DNase I is an endonuclease that cleaves (degrades) the phosphodiester bonds between nucleotides to digest DNA that has leaked into the dissociation medium as a result of cell damage during transfer and storage. Collagenase IV is a protease that cleaves the bonds between neutral amino acids (X) and glycine in the sequence Pro-X-Glyc-Pro, which is found with high frequency in collagen. Bovine Serum Albumin (BSA) stabilises the DNase and collagenase, and prevents adhesion of the enzymes to the tube during digestion. Fetal Calf Serum (FCS) and RPMI provide nutrients essential for cell growth such as glucose, amino acids, and growth factors.

Following centrifugation, the supernatant was removed and the pellet of cells was poured into a petri dish. 1ml of digestion mix was added to the petri dish and the tumour and colonic tissue were chopped very finely using a scalpel until no pieces were visible. The digestion mix was placed in a 50ml falcon tube, and the tumour and colonic tissue were transferred into the falcon tube. The tube was then placed horizontally on a shaking incubator at 70 rpm for 45 minutes at 37°C.

Following digestion, the tumour and tissue were filtered through 100 μm nylon mesh with HBSS.

Cell stimulation

In order to assess functional status of the specific immune cells of interest, cells were stimulated *in-vitro* with a standardised stimulation cocktail of PMA (phorbol myristate acetate), ionomycin and brefeldin A for 4 hours at 37°C. PMA activates protein kinase C which plays an important role in T cell activation. Ionomycin is a Ca^{2+} ionophore derived from the bacterium *Streptomyces Conglobatus*. It raises intracellular calcium levels, and is used in conjunction with PMA to activate T cells. PMA and ionomycin activate the transcription factors NF- κ B and NFAT leading to the production of the cytokines (IL-2, IL-10, IL-17). Brefeldin A is an antiviral metabolite produced by the fungus *penicillium brefeldanum*. It blocks transport of cytokines from the endoplasmic reticulum/Golgi apparatus. The synthesis and packaging of proteins into vesicles takes place in the endoplasmic reticulum, before transfer to the Golgi apparatus where they are dispatched intra and extracellularly. Inhibition of intracellular transport processes allows accumulation of proteins in the endoplasmic reticulum and enhances the staining signal.

Live cell staining

To ensure live cells were identifiable on analysis, a live/dead stain diluted 1:600 in Phosphate Buffered Saline (PBS) was added to the cells at a final volume of 50 μl per sample tube. Cell viability can be

measured by the binding of a dye to amines within the cell to determine if the cell membrane is intact. The dye in live/dead stains penetrates the cell membrane of dead cells and binds to DNA, resulting in very bright fluorescence. The dyes are unable to penetrate the membranes of live cells. Exclusion of dead cells from the data allows cleaner separation and identification of cell populations. Cells were incubated in the dark for 30 minutes on ice, washed with 1ml PBS and stained as described below.

Extracellular staining

Single cell suspensions were stained in FACS buffer for 30 minutes in the dark with fluorescently labelled monoclonal antibodies. A full list of antibodies can be found in Table 3. An extracellular fluorochrome-labelled antibody cocktail was prepared in FACS buffer (diluted 1:100), which contains Fc block to reduce non-specific binding of the antibody to Fc receptor (i.e. false positives). Cells were incubated in the dark with the extracellular-antibody master mix at a final volume of 50µl per tube for 25 minutes at room temperature.

Fixation and permeabilisation

Fixation and permeabilisation of cells is necessary prior to intracellular staining. This step preserves the cells by terminating all enzymatic activity and was performed using the CytoFast Fix/Perm kit (Biolegend). A paraformaldehyde is used to fix or cross-link cellular proteins, whilst a surfactant disrupts cell membrane by removing membrane lipids to allow the antibodies to enter the cell. Cells were

fixed by adding 100µl of intra-cellular fixation buffer to each tube for 30 minutes at 4°C. Cells were then washed with 1ml 1X permeabilisation buffer for intracellular antigen staining.

Intracellular staining

Following fixation and permeabilisation, an intracellular fluorochrome-labelled antibody cocktail was prepared in 1X perm buffer (diluted 1:100). Cells were incubated with this master mix at a final volume of 50µl per tube for 30 minute on ice. Cells were washed with 1X FACs buffer and re-suspended in 200µl of FACs buffer prior to analysis.

2.5.6 Data analysis

Samples were analysed on the BD Fortessa flow cytometer in Trinity College Dublin, and run to record a minimum of 100,000 events. Data analysis in flow cytometry is based on a process called gating. Gates are graphical regions or boundaries placed around populations of cells with common characteristics that enable in-depth assessment of these populations of interest. The first step in gating is to distinguish populations of cells based on their forward and side scatter properties. Forward and side scatter give an estimation of the size and granularity of the cells respectively. The next step is to remove dead cells. Once the live cells have been identified, further gates can be applied to determine the expression patterns on particular cell types. The data can be presented in the form of single parameter or two parameter density or contour plots. For

two parameter plots, a quadrant marker can be used to divide the plots into four sections to discriminate populations as negative, single positive or double positive. The upper left quadrant indicates cells that are only positive for the y-axis parameter. The lower left quadrant displays cells that are negative for both markers (double negative). The lower-right quadrant indicates cells that are positive for the x-axis marker but negative for the y-axis marker. The upper-right quadrant of the plot indicates cells positive for both markers (double positive).

2.6 Single cell RNA sequencing

I am extremely grateful and indebted to Cathal Harmon and Haim Moore in the Department of Medicine, Harvard Medical School, Boston, USA for their technical prowess and scientific expertise in performing single cell RNA sequencing. The samples were collected, prepared for storage and processing in the laboratory in Trinity College Dublin, and transferred to the Department of Medicine, Harvard Medical School for single cell RNA sequencing.

Principles

Traditional RNA-seq methods analysed the RNA of an entire population of cells, providing average gene expression of all cells, thereby missing subtle differences and masking heterogeneity.

Single cell RNA sequencing provides each individual cell's transcriptome and allows comprehensive analysis of cell subpopulations within a heterogeneous population. It can be used to identify rare cell populations that would otherwise go undetected in pooled analyses, or to analyse cellular responses by evaluating individual gene or protein expression.

The 10x Genomics technology uses microfluidic partitioning to capture single cells and prepare barcoded cDNA libraries. Single cells, reverse transcription reagents, Gel Beads containing barcoded oligonucleotides, and oil are combined on a microfluidic chip to form single cell Gel Beads in Emulsion (GEMs). A single cell is lysed within each GEM, the Gel Bead is dissolved to release the

identically barcoded oligonucleotides into solution, and reverse transcription of mRNA occurs. The cDNAs from a single cell have the same barcode, and can be mapped back to their original single cells of origin. Next generation sequencing (NGS) libraries are then prepared from these barcoded cDNAs.

Technique

Single cell RNAseq was performed on single cell suspensions of sorted T cells from 7 paired tumour and adjacent normal colon samples using the 10x Genomics platform. The samples were transferred to Harvard Medical School and the processing was performed by Cathal Harmon and Haim Moore. The samples were dissected into 1-2mm pieces and placed in CryoStor® cell cryopreservation media as previously described. Samples were then thawed and digested into single cell suspensions and fluorescence-activated cell sorting (FACS) was performed to select populations of T cells. Cell suspensions were barcoded (10x Chromium Single Cell platform controller, 10X Genomics) to generate single cell Gel Beads-in-Emulsion (GEMS). GEMs were then processed to generate Unique Molecular Identifiers (UMI)-based libraries according to the Chromium Single Cell 3' Library protocol. The 10X data was analysed using the Seurat platform and harmony integration in R.

2.7 Data collection and management

The studies detailed in this thesis were carried out in accordance with the principles of Good Clinical Practice. The protocols of all studies were reviewed and approved by the institutional research and ethics board.

Clinical data

Clinical data was retrieved from a prospectively maintained database of all patients with histologically confirmed colorectal cancer treated in St Vincent's University Hospital, Dublin.

Biological data

Patient samples were collected from patients undergoing surgery in St Vincent's University Hospital, Dublin. Informed consent was obtained prior to sample collection. All samples were coded with a unique pre-assigned study number. Data was stored on a password protected hard drive.

International collaborative

The Research in Early Age Colorectal Cancer Trends (REACCT) Collaborative was established in order to aggregate large volume, global data on early age onset colorectal cancer. The aim of the collaborative was to collect on a multi-institutional basis data relating to demographics, clinical characteristics, molecular and genetic profile, treatment methods (surgery, chemotherapy, radiotherapy), and outcome of patients diagnosed with EOCRC (defined as less than 50

years of age). It is hoped these data will help define age-specific screening, preventative, and therapeutic strategies, and identify unmet research needs.

Tertiary referral units worldwide with specialist expertise in CRC were invited to participate. An email with attached letter of invitation was sent to institutions across the globe via a designated REACCT collaborative email account. A REACCT Collaborative twitter account was also established to connect with as many institutions as possible. Any centre experienced in CRC was welcome to participate; there was no selection criteria related to prior experience or to the size of the centre. Additionally, a REACCT Collaborative logo was designed by a professional graphic designer. An image of a tree was chosen to represent youth, energy, and life. The person at the centre of the image symbolizes that patients are the focus of the collaborative.

A study protocol and pre-determined data set were sent to all participating institutions. Ethical approval was sought at individual institutional level. A principal investigator from each participating centre supervised data acquisition from an institutional database or independent review. Data entry, storage and high end data protection was via the REDCap system. REDCap is a protected digital cache with advanced electronic security measures. The REACCT Collaborative REDCap database was managed by the REDCap team in the Centre for Clinical Research in St. Vincent's University

Hospital. The data retrieved was fully anonymized and it was not possible for any patients in the dataset to be traced.

The REACCT Collaborative currently has 135 member institutions in 43 countries across every inhabited continent. Data submitted by participating centres was aggregated in the REDCap database from which patients were identified for the various studies described in this thesis. Data on a total of 4398 patients were collected.



Figure 3. REACCT Collaborative logo



Figure 4. Geographical representation of participating institutions of the REACCT Collaborative

2.8 Data analysis

Statistical Analysis

Continuous variables were described as mean ($\pm$ standard deviation) or median values (range) and compared by the Student t test or Mann-Whitney U test, depending on their distribution, Categorical variables were reported as percentages. Association of categorical variables was assessed using χ^2 test or Fisher exact test where appropriate. Survival statistics were calculated using the Kaplan-Meier method, and the log-rank test was used to assess differences in survival between groups. Independent variables were entered into a multivariable binary logistic regression model. Variables that were found at univariable analysis to be significant, or a p-value <0.1 were entered into the multivariable model. A significance level of 0.05 was used for all analyses; reported p-values are 2-tailed. Data were analyzed using SPSS® software version 24.0 (IBM, Armonk, New York, USA).

Chapter 3: Clinicopathological features and oncological outcomes of patients with early age onset rectal cancer

Abstract

3.1 Background

3.2 Methods

3.2.1 Study population

3.2.2 Treatment protocol

3.2.3 Pathological analysis

3.2.4 Study endpoints

3.2.5 Statistical analysis

3.3 Results

3.3.1 Patient demographics, clinical characteristics and pathological features

3.3.2 Survival

3.3.3 Disease recurrence

3.3.4 Univariable and multivariable analyses of factors that influence survival

3.4 Discussion

Abstract

Background: The incidence of rectal cancer in adults under the age of 50 is increasing. Survival data are limited, and the oncological benefit of conventional neoadjuvant and adjuvant therapies is unknown.

Methods: Disease-specific outcomes of patients diagnosed with rectal cancer undergoing surgical resection with curative intent between 2006 and 2016 were analysed. Patients aged less than 50 were compared to those aged 50 and above.

Results: A total of 797 patients with rectal cancer were identified of whom 685 underwent surgery with curative intent. Seventy were aged under 50 years and 615 were aged 50 years or over. There was no difference in clinical stage between the two age groups. Patients aged less than 50 were more likely to have microsatellite instability (9% vs. 2%, $p=0.003$) and Lynch Syndrome (7% vs. 0%, $p<.001$). Overall 5-year survival was better in those aged less than 50 (80% and 72%; $p=0.013$). Disease-free 5-year survival was 81% in both age groups ($p=0.711$). The development of locoregional recurrence or distant metastases did not differ between groups. Younger patients were more likely to receive neoadjuvant chemoradiotherapy (67% vs. 53%, $p=0.003$) and adjuvant chemotherapy (41% vs. 24%, $p=0.006$).

Conclusion: Young patients have comparable disease-specific outcomes to older counterparts, despite receiving more neoadjuvant and adjuvant treatment. MSI and a diagnosis of Lynch Syndrome were more common in early age onset disease.

3.1 Background

The incidence of colorectal cancer (CRC) among young adults under the age of 50 years has risen dramatically worldwide. This trend is predominantly driven by an increase in rectal cancer ²¹⁹. Within the next decade, it is estimated that almost 25% of rectal cancers will be diagnosed in individuals aged less than 50 ⁹. The reasons for this rise are unknown. Although early age onset disease is more likely to arise in the context of a hereditary cancer syndrome, the majority of cases are sporadic displaying considerable genotypic and phenotypic heterogeneity.

Younger patients typically present with more advanced disease and adverse histopathological features than older counterparts yet have comparable (or better) short- and long-term survival ^{136,220-224}. The underlying biomolecular profile of early age onset rectal cancer remains undefined. It is possible the disease process is driven by alternative oncogenic pathways to later onset disease. Furthermore, the sensitivity of early age onset CRC to standard neoadjuvant and adjuvant therapies is unclear as young patients have historically accounted for a small proportion in clinical trials. The aim of this study was to analyse the clinical and pathological features, long-term survival and disease recurrence patterns among patients diagnosed with rectal cancer aged less than 50 years and to compare cancer-specific outcomes to patients aged 50 years and older.

3.2 Material and Methods

3.2.1 Study population

A prospectively registered consecutive series of patients with histologically confirmed rectal cancer undergoing surgery with curative intent at St Vincent's University Hospital, Dublin, between 2006 and 2016 were retrospectively studied. Rectal cancer was defined as adenocarcinoma within 15cm from the anal verge on colonoscopy. Clinical staging was according to the 8th Edition of the American Joint Committee on Cancer tumour node metastasis (TNM) staging system and was based on a combination of pelvic MRI and CT thorax, abdomen and pelvis. Baseline demographic, clinical, staging, treatment, histopathologic and survival data were identified on a prospectively maintained database.

3.2.2 Treatment protocol

All patients were discussed at the institutional multidisciplinary team meeting following histological diagnosis and radiological staging. Patients with clinical stage 3 disease or aggressive stage 2 disease (extramural venous invasion, T4 disease, threatened mesorectal margin) received long-course neoadjuvant chemoradiotherapy (45-50.4 Gy delivered in daily fractions over 5-6 weeks and concurrent 5-FU based chemotherapy). Following completion of neoadjuvant therapy, patients were restaged by CT of thorax, abdomen and pelvis, MRI pelvis and clinical/endoscopic evaluation. If there was no evidence of local or systemic disease progression and performance status had not deteriorated significantly, total mesorectal excision was

performed after an interval of 10-12 weeks. Adjuvant chemotherapy was routinely given to patients with predicted stage 3 and those with histological node positive disease who were otherwise fit. For patients with Stage 2 disease, adjuvant chemotherapy was considered on a case-by-case basis following multidisciplinary discussion.

All patients were followed up with an annual CT scan and endoscopy at 1, 3, and 5 years or where clinically indicated. Locoregional recurrence was defined as that occurring at the site of the anastomosis or within the pelvis. Distant recurrence was defined as that occurring within a solid organ. Cytologic, histologic, or radiologic proof was required to confirm a diagnosis of recurrent disease.

3.2.3 Pathological analysis

The tumour stage was defined according to the TMN staging system and the American Joint Committee on Cancer classification.

Haematoxylin and eosin sections of the resected specimen were analysed using a minimum dataset and a standardized reporting system. Microscopically clear resection (R0) was defined as a tumour-free resection margin of at least 1 mm. The absence of residual tumour cells in the resected specimen was defined as a complete pathologic response (pCR). Extent of residual carcinoma was assigned to one of three categories: TRG 1 represents no identifiable residual cancer cells (pCR), TRG 2 represents residual cancer outgrown by fibrosis and TRG 3 represents significant fibrosis outgrown by cancer or no fibrosis with extensive residual cancer²²⁵. Microsatellite instability

was assessed in all patients using immunohistochemistry for mismatch repair proteins, MLH1, PMS2, MSH2, and MSH6. Germline testing was performed as needed following patient counseling and consent.

3.2.4 Study endpoints

The primary endpoints were overall and disease-free survival.

3.2.5 Statistical analysis

Data were analyzed using SPSS software Version 24.0. A significance level of 0.05 was used for all analyses; reported p-values are 2-tailed. Continuous variables are described as mean ($\pm$ standard deviation) or median values (range) and compared by the Student's t test or Mann-Whitney U test, depending on their distribution. Categorical variables are reported as percentages. Association of categorical variables was assessed using χ^2 test or Fisher exact test where appropriate. For follow-up data, date of death or last follow-up was entered. Disease-free survival (DFS) and overall survival (OS) rates were calculated according to the Kaplan-Meier method and group comparisons were based on the log-rank test. Independent variables were entered into a multivariable Cox proportional hazards regression model. Only variables that were found at univariable analysis to be significant were entered into the multivariable model.

3.3 Results

3.3.1 Patient demographics, clinical characteristics and pathological features

Between 2006 and 2016, 797 consecutive patients were diagnosed with rectal cancer. Metastatic disease was present at diagnosis in 87; 10 (12%) aged under 50 and 77 (13%) aged 50 or over. Of those with non-metastatic disease, 685 patients underwent surgery with curative intent and comprise the study group. Of those, 70 were aged under 50 years and 615 were aged 50 years and above. Clinicopathological characteristics of the study population are summarised in Table 4. Age less than 50 years was associated with microsatellite instability and diagnosis of Lynch Syndrome, but was not associated with clinical stage, pCR, R0 resection rate, or pathological stage. Young patients were more likely to undergo pelvic exenteration, and to receive neoadjuvant and adjuvant therapy.

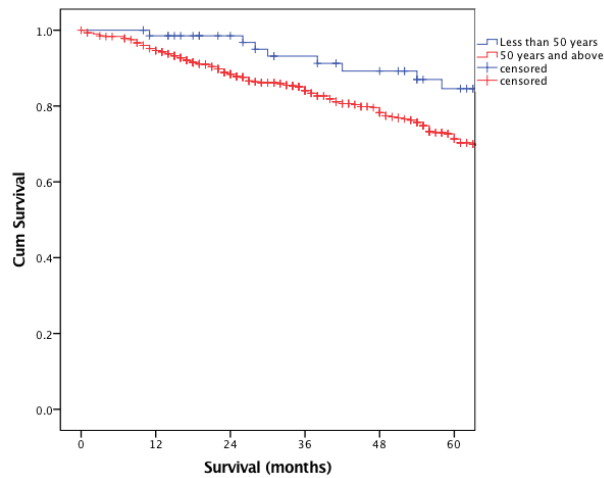
Table 4. Comparison of demographics and clinicopathological features according to age

	<50 years (N = 70)	≥50 years (N = 615)	<i>p</i> value
Demographics			
Male n (%)	40 (57)	393 (64)	0.295
cStage			
I/II	23 (33)	218 (35)	0.298
III	47 (67)	324 (53)	0.302
Unknown	0 (0)	73 (12)	
Neoadjuvant CRT, n (%)	47 (67)	328 (53)	0.031
Type of Operation			
Anterior Resection	54 (77)	425 (69)	0.101
AP Resection	9 (13)	99 (16)	0.604
Transanal excision	2 (3)	59 (10)	0.074
Pelvic exenteration*	4 (6)	8 (1)	0.026
Other (e.g. Hartmann's)	1 (1)	24 (4)	0.501
(y)pTNM stage			
I	14 (20)	172 (28)	0.201
II	18 (26)	157 (25)	1.000
III	31 (44)	221 (36)	0.239
Pathology			
pCR, n (%)	7 (10)	62 (19)	0.687
R0 resection, n (%)	67 (96)	585 (95)	1.00
MMR status			
MMRd	6 (9)	10 (2)	0.003
Lynch Syndrome	5 (7)	0 (0)	<0.001
Adjuvant Chemotherapy	29 (41)	149 (24)	0.006

cStage = clinical stage according to AJCC. *All patients undergoing pelvic extenteration had T4 disease

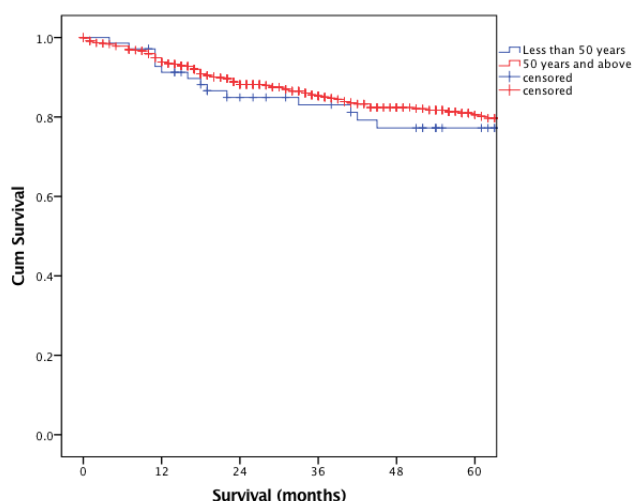
3.3.2 Survival

The overall median (range) follow-up was 48 (1-169) months. In patients less than 50, the median overall survival was 60 months (10-166), with 1, 3 and 5-year overall survival 96%, 88% and 80% respectively. In the ≥ 50 group, the equivalent was 44 months (3-169) and 95%, 85% and 72% ($p=0.013$). The median disease free survival was 54 months (7-166) in the less than 50 group and 38 months (7-169) in the ≥ 50 group. DFS at 1, 3 and 5-years was 96%, 87% and 81% in the under 50s compared with 95%, 85% and 81% ($p=0.711$) in the ≥ 50 group. Overall and disease-free survival data are presented in *Figures 5 and 6* respectively.



	Numbers at risk			
	0 months	12 months	36 months	60 months
< 50 years	70	63	48	32
≥ 50 years	615	523	340	194

Figure 5. Kaplan-Meier curve of overall survival for patients with rectal cancer aged less than 50 years vs. aged 50 years and above. Patients under 50 had better overall survival than those aged 50 and above (Log rank test, $p = 0.013$).



	Numbers at risk			
	0 months	12 months	36 months	60 months
< 50 years	70	58	43	29
≥ 50 years	615	492	302	171

Figure 6. Kaplan-Meier curve of disease-free survival for patients with rectal cancer aged less than 50 years vs. aged 50 years and above. There was no significant difference in disease-free survival between patients aged less than 50 and those aged 50 and above (Log rank test, $p = 0.711$)

3.3.3 Disease recurrence

Disease recurrence occurred in 15 patients (21%) aged less than 50 years and in 102 patients (17%) aged 50 years or above ($p=0.313$). Five patients (7%) in the under 50s developed locoregional recurrence compared to 30 patients (5%) in the 50 and above group ($p=0.573$). Ten patients (14%) aged under 50 years developed distant disease developed compared with 74 patients (12%) aged ≥ 50 years ($p=0.567$). The median time to recurrence was 21 months (7-157) from surgery in the less than 50 group and 19 months (7-103) in the 50 and over group.

3.3.4 Univariable and multivariable analyses of factors that influence survival

On univariable analysis, the only variable in the under 50 group associated with better disease-specific survival (DSS) was R0 resection. In the 50 and above group, nCRT, pCR, R0 resection and node negativity were associated with better DSS. On multivariable analysis, R0 resection and (y)pN0 were significantly associated with DSS in the 50 and over group (HR 3.435, CI 1.930 - 6.112, $p = <0.001$ and HR 1.744, CI 1.071 – 2.839, $p = 0.025$ respectively). Age-based univariable logistic regression of factors predicting DSS is presented in Table 5.

Table 5. Age-based Univariable Logistic Regression of Factors Predicting Disease Specific Survival

Variable	< 50 years			≥ 50 years		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Age (years)	1.014	.919-1.119	0.782	1.012	.991-1.033	0.251
Gender (male)	0.638	.218-1.869	0.413	1.249	.841-1.857	0.270
cStage I/II	0.663	.210-2.088	0.482	0.875	.574-1.334	0.535
cStage III	1.509	.479-4.755	0.482	1.21	.794-1.843	0.376
nCRT	1.955	.551-6.931	0.299	1.897	1.243-2.89	0.003
TRG 2/3	0.562	.072-4.355	0.581	0.339	.147-.782	0.011
R0 resection	4.924	1.106-21.929	0.036	5.319	3.180-8.897	<0.001
Node negative	2.892	.987-8.478	0.053	2.093	1.416-3.095	<0.001
MSI	1.717	.387-7.616	0.477	0.970	.239-3.936	0.966
Adjuvant CT	2.278	.809-6.409	0.119	1.465	.946-2.269	0.087

cStage = clinical stage according to AJCC. TRG = tumour regression grade. MSI = microsatellite instability. CT = chemotherapy

3.4 Discussion

Data from large registry based studies in Europe and the United States, indicate that rectal cancer rates have increased in adults aged less than 50 years over the past few decades⁸. The greatest annual percentage change is between 20 to 34 years^{9,226}. There is an urgent need to understand the disease process in order to inform effective therapeutic strategies.

Survival data for early onset disease are conflicting. Several studies indicate a worse prognosis whilst others demonstrate equivalent or better oncologic outcomes^{114,136,224,227}. In this series, young patients displayed equivalent short- and long-term disease-specific survival to their older counterparts. Rates of locoregional recurrence and distant disease failure was also similar between groups. Notably, the under 50s were more likely to receive neoadjuvant and adjuvant therapy.

Although young patients are more likely to receive neoadjuvant chemoradiotherapy than older patients, the oncological benefit remains to be defined. Patients aged under 50 years accounted for a small percentage overall in the landmark clinical trials that shaped rectal cancer management^{47,60,61}. Recent data suggest that multimodal therapy for stage II and III disease may not be associated with an overall survival benefit in patients aged under 50 years¹³⁶. It is noteworthy that two-thirds of the under 50s in the current series presented with predicted node positive disease, compared to half of the over 50s. Interestingly, the pCR rate was lower in the under 50s.

Although not statistically significant, older patients were almost twice as likely to achieve a pCR. These findings may be relevant when considering an organ preservation approach in young patients. Despite a higher pCR rate in the over 50s, there was no significant difference in local recurrence between the two groups. The number of patients in the analysis is too low to draw any definite conclusions but the data certainly raise important questions surrounding neoadjuvant therapy, pathological response and non-operative management in young patients with rectal cancer.

Young patients were also more likely to receive adjuvant chemotherapy. Adjuvant chemotherapy has in the past been administered in locally advanced disease due to better oncological outcomes²²⁸⁻²³⁰. Its role in the modern era of neoadjuvant therapy is less clear. The rigorous systemic treatment of patients with early onset disease may be reflective of physician attitude. High performance status and the perception of aggressive disease may influence therapeutic decision making. In addition, physicians may be fearful of undertreating young patients due to their longer life expectancy and potentially greater loss of life-years. Nonetheless, it is important to consider chemotherapy-related toxicity and overtreatment of patients with low risk disease in the absence of a definitive oncological benefit⁶³⁻⁶⁶. Deciphering the molecular signature of the tumour will be instrumental in refining and optimising treatment strategies.

In addition to receiving more neoadjuvant and adjuvant therapy, young patients were more likely to undergo pelvic exenteration. This finding may reflect better performance status, but also surgeon attitude. Surgical decision making may be influenced by the anticipated survival benefit and life years gained. A careful balance between quantity and quality of life should be sought, as major pelvic surgery can be associated with significant long-term morbidity.

Early age at disease onset is a hallmark of inherited cancer predisposition. In this series, age less than 50 years was significantly associated with microsatellite instability and a diagnosis of a hereditary cancer syndrome. Despite the increased likelihood of a genetic cause in the under 50 group, only 7% were diagnosed with Lynch Syndrome. These data support the hypothesis that the increase in young rectal cancer is due to a rise in sporadic disease the driver of which remains undefined. Nonetheless, deciphering the genetic component of early onset disease is important to guide treatment and identify at risk family members. As the spectrum of genes implicated in hereditary CRC expands, multigene panels are increasingly being used to test for a variety of hereditary cancer syndromes ¹³⁰. This expanded testing is of particular importance as a significant proportion of patients with germline mutations do not report a CRC diagnosis in a first degree relative ¹³⁴.

This study has limitations including the single institution, retrospective nature, and dichotomisation by age using an arbitrary

integer cut-off (50). Survival outcomes differ among patients under 50 years (independent of disease stage) supporting further age-based subgrouping⁹⁹. Due to relatively small numbers in the under 50 group, further subgrouping was not possible in this analysis. As the epidemiology of CRC evolves, current population-based screening strategies may need to be refined. The American Cancer Society have recommended lowering the age of initial screening from 50 to 45 years^{231,232}. However, given that the greatest change in incidence is among those aged 20-39 years, optimal population education is a healthcare priority.

Chapter 4. An international multicentre study of clinical characteristics, pathological features and oncological outcomes of patients with early age onset colorectal cancer

Abstract

4.1 Background

4.2 Methods

4.2.1 Study participants

4.2.2 Data collection

4.2.3 Treatment

4.2.4 Pathology

4.2.3 Study endpoints

4.2.4 Statistical analysis

4.3 Results

4.3.1 Baseline demographics

4.3.2 Clinical characteristics

4.3.3 Neoadjuvant and adjuvant therapy

4.3.4 Pathology

4.3.5 Molecular features

4.3.6 Oncological outcomes

4.3.7 Univariable and multivariable analyses of factors that influence survival

4.4 Discussion

Abstract

Background: There is a worrying rise in colorectal cancer (CRC) incidence in the under 50 age group. Initial outcome data were limited by the relative scarcity but, as experience is growing, distinct clinicopathological patterns are emerging. This aim of this study was to define these features and cancer-specific outcomes in patients with CRC under the age of 50 years.

Methods: Anonymized data from an international collaboration was collected. All patients under the age of 50 with stage I-III CRC in each institution diagnosed between 2000-2020 were included. The primary study outcomes were clinicopathological features and disease-free survival.

Results: Data on a total 3378 patients (median age 43 with 54.3% male) was entered with distal disease (sigmoid and rectum) accounting for 70.1%. Neoadjuvant therapy was administered to 160 (34.8%), 173 (42.1%) and 194 (31.1%) of those with stage I, II and III rectal cancer. Among patients with colon cancer, 215 (34.3%) and 597 (73.7%) of those with stage II and III disease respectively received adjuvant chemotherapy. The corresponding proportions for patients with rectal cancer were 126 (30.7%) and 419 (67.3%). Pathological stage III disease was diagnosed in 810 (43.0%) patients with colon cancer and in 623 (41.7%) patients with rectal cancer. Microsatellite instability was present in 20.0% (1 in 4 colon cancers and 1 in 10 rectal cancers), of whom 71 (33.6%) were diagnosed with a definable hereditary cancer syndrome. Five-year disease-free survival for stage I, II, and III colon cancer was 96%, 91%, and 68% respectively. The

equivalent rates for patients with rectal cancer were 91%, 81%, and 62%.

Conclusions: EOCRC is more likely to involve the distal colon and rectum, demonstrate MSI, and be associated with a genetic predisposition compared to later-onset disease. Simple screening with limited endoscopy and targeted molecular therapy may improve survivorship.

4.1 Background

Although the overall incidence and mortality of colorectal cancer (CRC) has decreased globally since the introduction of population-based screening, there has been a significant rise in incidence among adults aged less than 50 years. Early age onset CRC (EOCRC) now represents the second most common cancer and the third leading cause of cancer-related death in this age group¹⁰². Similar trends have been observed in Europe and North America with the greatest increase in distal colon and rectal cancers^{8,9,100,233}. Analysis of 143 million people from 20 European countries showed CRC incidence has increased by 7.9% per year among subjects aged 20–29 years, by 4.9% among those aged 30–39 years, and by 1.6% among those aged 40–49 years from 2004 to 2016⁸. Based on current data, it is estimated that within the next decade 1 in 4 rectal cancers and 1 in 10 colon cancers will be diagnosed in individuals under 50⁹.

The factors driving these escalating trends are unclear. Although more likely to occur in the context of a hereditary cancer syndrome, the majority of EOCRCs are sporadic^{129–131}. Environmental and lifestyle factors such as diet, cigarette smoking, and alcohol consumption in isolation do not explain the observed trends. Importantly, EOCRC represents a global phenomenon and affects both genders. Potential risk factors hypothesised include obesity, infections, antibiotics, and alterations to the gut microbiome¹²⁶.

Some of the clinicopathological characteristics of EOCRC differ to those of late-onset disease. EOCRC typically involves the distal colon or rectum, presents at advanced disease stage and displays unfavourable histopathological features such as poor differentiation, mucin and signet-ring morphology¹¹¹⁻¹¹³. Survival data is lacking and conflicting, and despite increased use of neoadjuvant/adjuvant treatment among patients with EOCRC sensitivity to conventional chemo(radio)therapy is unknown^{113,135,136}. Unique tumour biology may influence response to treatment, and age at diagnosis is not considered in modern therapeutic strategies. The long-term effects of chemo/radiotherapy on quality of life and fertility are of particular importance in this patient group.

Although increasing in incidence, EOCRC accounts for a very small proportion of CRCs overall. Individual institutional data alone is of limited value in addressing the big questions that surround this disease. The REACCT Collaborative was established to aggregate large volume “real-world” data from specialist centres across the world. The aim of this study was to determine the clinicopathological features and cancer-specific outcomes of patients diagnosed with CRC under the age of 50 years.

4.2 Methods

4.2.1 Study participants

A retrospective international multicentre observational cohort study to assess the clinicopathological features and oncological outcomes of patients diagnosed with EOCRC over a 20-year period (2000 to 2020) was performed. Inclusion criteria were adults aged between 18 and 49 years with a histologically confirmed diagnosis of CRC who underwent surgery with curative intent. Patients with distant metastases at diagnosis were excluded.

4.2.2 Data collection

Patients who fulfilled the inclusion criteria of the study were identified from the REACCT Collaborative database. A principle investigator in each institution was responsible for data collection. Ethical approval was obtained at institutional level. Collected data included patient demographics, neoadjuvant therapy, surgical intervention, histopathological features, surgical outcomes, adjuvant therapy and cancer-specific as well as overall survival information. Rectal cancer and tumours of the colon were evaluated separately. Rectal cancer was defined as tumours within 15m from the anal verge on colonoscopy. Clinical staging was according to the 8th Edition of the American Joint Committee on Cancer (AJCC) tumour node metastasis (TNM) staging system.

4.2.3 Treatment

All participating institutions were tertiary referral centres with expertise in CRC. Management of colonic and rectal tumours was in accordance with international guidelines and best practice, and following multidisciplinary management. In general, upfront segmental colectomy was performed in patients with colon cancer. Neoadjuvant chemoradiotherapy was given to patients with locally advanced rectal cancer (clinical stage III or stage II with aggressive features e.g. T4 disease, threatened mesorectal margin). In general, patients received either long-course neoadjuvant chemoradiotherapy (45-50.4 Gy delivered in daily fractions over 5-6 weeks and concurrent 5-FU based chemotherapy) or short-course radiotherapy (5.0 Gy over 5 days without chemotherapy). Adjuvant fluorouracil-based chemotherapy (for both colon and rectal tumours) was administered to patients with node positive disease, or to patients with stage II disease with high risk features who had a good performance status.

4.2.4 Pathology

As described in Methods, the tumour stage was defined according to the TMN staging system and the American Joint Committee on Cancer classification. Haematoxylin and eosin sections of the resected specimen were analysed using a minimum dataset and a standardized reporting system. Microscopically clear resection (R0) was defined as a tumour-free resection margin of at least 1 mm. The absence of residual tumour cells in the resected specimen was defined as a

complete pathologic response (pCR). Extent of residual carcinoma was assigned to one of three categories: TRG 1 represents no identifiable residual cancer cells (pCR), TRG 2 represents residual cancer outgrown by fibrosis and TRG 3 represents significant fibrosis outgrown by cancer or no fibrosis with extensive residual cancer ²²⁵. Microsatellite instability was identified by PCR or by loss of mismatch repair proteins, MLH1, PMS2, MSH2, and MSH6 on immunohistochemistry. The definition of hereditary cancer syndrome was diagnosis of a constitutive pathogenic variant on germline testing.

4.2.5 Study endpoints

The primary endpoints were overall and disease-free survival. Secondary endpoints for rectal cancer were pathological response rates and the impact of neoadjuvant and adjuvant therapy on survival. For colon cancer, the secondary endpoint was the impact of adjuvant therapy on survival.

4.2.6 Statistical Analysis

Continuous variables were described as mean ($\pm$ standard deviation) or median values (range) and compared by the Student t test or Mann-Whitney U test, depending on their distribution, Categorical variables were reported as percentages. Association of categorical variables was assessed using χ^2 test or Fisher exact test where appropriate. Survival statistics were calculated using the Kaplan-Meier method, and the log-rank test was used to assess differences in survival between groups. Independent variables were entered into a multivariable binary logistic

regression model. Variables that were found at univariable analysis to be significant, or a p-value <0.1 , were entered into the multivariable model. A significance level of 0.05 was used for all analyses; reported p-values are 2-tailed. Data were analyzed using SPSS® software version 24.0 (IBM, Armonk, New York, USA).

4.3 Results

4.3.1 Baseline Demographics

A total of 3,378 patients diagnosed with non-metastatic colorectal cancer under the age of 50 over a 20-year interval were included in the study. The median (range) age was 43 (18-49) years and 1835 (54.3%) were male. Median (range) BMI was 24 (12-59). The majority of patients were white Caucasian (45.2%) or Asian (29.6%). Never smokers accounted for 67% and 15% had a history of excess alcohol consumption defined as >14 standard units/week. Most patients were well with an American Society of Anaesthesiologists (ASA) grade of 1 or 2 (92.4%) and ECOG performance status of 0 or 1 (98.1%). Only 3.2% (n=109) had a diagnosis of inflammatory bowel disease, of which ulcerative colitis was most common (74%). Almost one third (31.8%) had a first degree relative with CRC. None had a confirmed hereditary cancer syndrome prior to diagnosis. Baseline demographics are summarised in *Table 6*.

4.3.2 Clinical Characteristics

Rectal tumours accounted for 44.2% (n=1494), while 1884 patients had tumours located in the colon. The majority of colonic tumours involved the sigmoid colon (n= 674, 35.8%). Synchronous tumours were uncommon (0.8%). Diagnosis was an incidental finding in 184 patients (5.4%). Clinical characteristics are summarised in *Table 6*.

Table 6. Demographics and clinicopathological data of patients with colonic and rectal cancer

	Colonic N = 1884	Rectal N = 1494
Median age (range)	43 (18-49)	43 (18-49)
Male	997 (52.9%)	838 (56.1%)
Median BMI (range)	24 (13-59)	23 (12-50)
Inflammatory Bowel Disease	67 (3.6%)	42 (2.8%)
First Degree Relative with CRC	591 (31.4%)	482 (32.3%)
Known hereditary cancer syndrome	0 (0%)	0 (0%)
Symptoms		
Abdominal pain	580 (30.8%)	174 (11.6%)
Change in bowel habit	393 (20.9%)	380 (25.4%)
Rectal bleeding	535 (28.4%)	873 (58.4%)
Anaemia	235 (12.5%)	51 (3.4%)
Incidental finding	132 (7.0%)	52 (3.5%)
Other	170 (9.0%)	88 (5.9%)
Tumour site		
Rectosigmoid junction	202 (10.7%)	-
Sigmoid colon	674 (35.8%)	-
Descending colon	160 (8.5%)	-
Splenic flexure	121 (6.4%)	-
Transverse	191 (10.1%)	-
Hepatic flexure	58 (3.1%)	-
Ascending colon	252 (13.4%)	-
Caecum	239 (12.7%)	-
Synchronous tumour	13 (0.6%)	14 (0.9%)
Neoadjuvant therapy	58 (3.0%)	638 (42.7%)
Type of neoadjuvant therapy		
Chemoradiotherapy	-	526 (35.2%)
Radiotherapy only	-	72 (4.8%)
Chemotherapy only	58 (3.0%)	40 (2.7%)
pStage		
I	448 (23.8%)	460 (30.8%)
II	626 (32.2%)	411 (27.5%)
III	810 (43.0%)	623 (41.7%)
Adjuvant chemotherapy	885 (47.0%)	691 (46.3%)

pStage = pathological stage according to AJCC.

4.3.3 Neoadjuvant and adjuvant therapy

Neoadjuvant (chemo)radiotherapy was administered to 638 (42.7%) patients with rectal cancer. The median radiotherapy dose was 50Gy and the most common chemotherapy agent administered was capecitabine. Neoadjuvant chemotherapy was administered to 58 (3.0%) patients with colon cancer. Adjuvant chemotherapy was given to 885 (47.0%) and 691 (46.3%) of patients with colon and rectal cancer respectively. FOLFOX and CAPOX were the most commonly administered regimes. Summarised in *Table 7*.

Table 7. Neoadjuvant and adjuvant therapy according to disease stage

	Stage I	Stage II	Stage III
Rectal cancer			
Neoadjuvant CRT	160 (34.8%)	173 (42.1%)	194(31.1%)
Adjuvant chemotherapy	70 (15.2%)	126 (30.7%)	419(67.3%)
Colon cancer			
Adjuvant chemotherapy	9 (2.0%)	215 (34.3%)	597(73.7%)

CRT = chemoradiotherapy

4.3.4 Pathology

Overall, an R0 resection was achieved in 3126 (92.5%) of patients: 1728 colon and 1398 rectal. Lymphovascular invasion, extramural invasion, and perineural invasion were present in 34.6%, 29.9%, and 19.4% of colon cancers and 33.0%, 22.6%, and 21.1% of rectal cancers respectively. A pCR was identified in 171 (26.8%) of patients with rectal cancer who received neoadjuvant therapy.

4.3.5 Molecular features

MSI was identified in 165 (27.0%) patients with colon cancer. Sixty patients (36.4%) with MSI tumours were diagnosed with a definable hereditary cancer syndrome. In patients with rectal cancer, 46 (10.9%) demonstrated MSI. Eleven patients (24.0%) were diagnosed with a definable hereditary cancer syndrome. Notably, 58 (35.2%) of those with MSI colon cancer and 14 (30.4%) of those with MSI rectal cancer, had not had genetic testing at the time of data collection.

4.3.6 Oncological outcomes

Overall median follow-up was 32 (1-240) months.

Colon cancer

The 5-year OS rates were 99%, 94%, and 82% for stage I, II, and III respectively. The 5-year DFS rates were 96%, 91%, and 68% for stage I, II, and III. Disease recurrence occurred in 247 patients (17.9%); 56 (4.1%) and 191 (13.8%) developed locoregional and systemic recurrence.

Rectal cancer

The 5-year OS rates were 98%, 90%, and 76% for stage I, II, and III respectively. The 5-year DFS rates were 91%, 81%, and 62% for stage I, II, and III. Disease recurrence occurred in 265 patients (21.2%); 83 (6.6%) developed locoregional recurrence and 212 (17.0%) developed systemic recurrence.

4.3.7 Factors influencing survival

R0 resection and pN0 status were significantly associated with better DFS survival in both colon and rectal cancer. Univariable and multivariable analysis are summarised in *Table 8 and 9*.

Table 8. Univariable and multivariable logistic regression of factors predicting disease free survival in patients with colonic cancer stage I-III

Variable	<u>Univariable</u>			<u>Multivariable</u>		
	HR	95% CI	p	HR	95% CI	p
Age (years)	1.007	0.986, 1.028	0.522	-	-	-
Male	0.750	0.567, 0.989	0.041	-	-	-
cStage I-II	2.524	1.717, 3.710	<0.0001	-	-	-
cStage III	0.396	0.270, 0.582	<0.0001	-	-	-
R0	3.647	2.144, 6.203	<0.0001	3.681	1.640, 8.263	0.002
pN0 status	5.084	3.723, 6.942	<0.0001	3.209	1.835, 5.611	<0.0001
MSI	0.610	0.350, 1.062	0.081	-	-	-

cStage = clinical stage according to AJCC

Table 9. Univariable and multivariable logistic regression of factors predicting disease free survival in patients with rectal cancer stage I-III

Variable	<u>Univariable</u>			<u>Multivariable</u>		
	HR	95% CI	p	HR	95% CI	p
Age (years)	0.986	0.967, 1.006	0.173	-	-	-
Male	0.918	0.705, 1.195	0.525	-	-	-
cStage I-II	2.263	1.263, 1.643	<0.0001	-	-	-
cStage III	0.399	0.287, 0.555	<0.0001	-	-	-
R0	1.001	1.007, 1.013	0.048	1.016	1.001,1.030	0.036
pN0 status	2.749	2.092, 3.614	<0.0001	3.187	1.660-6.121	<0.0001
MSI	0.375	0.143, 0.984	0.046	-	-	-

cStage = clinical stage according to AJCC

Impact of neoadjuvant and adjuvant therapy on survival

Neoadjuvant therapy did not improve DFS in patients with pathological (yP) node negative (log rank 0.92, $p = 0.338$) or positive rectal cancer (indeed it was associated with worse disease-free survival in the latter - log rank 11.95, $p = 0.001$). This was largely due to persistently positive disease despite receiving up-front therapy (i.e. poor tumour regression). DFS was modestly better in those patients who received adjuvant chemotherapy in stage III colon cancer (log rank 2.84, $p = 0.092$) – a larger sample size would be needed to achieve statistical significance. Meanwhile, the need for adjuvant chemotherapy in stage II colon cancer was associated with worse DFS

(log rank 6.02, $p = 0.014$). In rectal cancer patients who received adjuvant chemotherapy, similar DFS was observed for stage II (log rank 0.56, $p = 0.760$) and III (log rank 1.08, $p = 0.299$) disease.

4.4 Discussion

The epidemiology of CRC has changed significantly over the past three decades. Many questions exist surrounding the aetiology, molecular profile and genetic component of the disease, and large volume data specific to this patient group are lacking. This study presents the clinicopathological features and oncological outcomes of 3,378 patients diagnosed with non-metastatic CRC aged less than 50.

In keeping with previously published data^{4,9,104,234}, the majority of tumours (70%) were located in the sigmoid colon and rectum. Right and left sided cancers differ in terms of histopathology, molecular landscape, response to treatment, and recurrence patterns^{201,202}. Why young adults are disproportionately affected by distal colon and rectal cancer is unclear, and deciphering the aetiology of rectal versus colon cancer will be key to developing preventative strategies. Importantly, tumour location also guides optimum screening methods. Flexible sigmoidoscopy should capture the majority of EOCRC, representing a potential screening tool.

EOCRC is more frequently associated with adverse histopathological features and more advanced disease at diagnosis relative to late-onset disease^{103,112,113}. In this study, the proportions of patients with lymphovascular invasion, extramural venous invasion, perineural invasion, and node-positive disease were higher compared to those reported in older patients^{118,235-237}. Advanced disease stage may reflect aggressive tumour biology and/or be due to a delay from symptom

onset to diagnosis, either patient or physician related^{115,117}. The vast majority of patients were symptomatic, highlighting the importance of educational initiatives to raise awareness among young adults, primary care physicians and clinicians, and ensure timely diagnosis and intervention.

Survival data for patients with EOCRC is conflicting. Despite advanced disease stage and unfavourable histopathological features some studies report equivalent oncological outcomes, whilst others report better survival among young patients^{221,224,235,238,239}. In the present study, the survival data are comparable to those of stage-matched older patients.

Standard oncological principles of surgical resection apply to patients with EOCRC. Better pre-operative bowel function and continence in young patients may allow for more radical sphincter-preserving techniques such as colo-anal anastomosis to enable avoidance of a permanent stoma²²¹. In patients with sporadic disease, extended resections have not resulted in better disease-free or overall survival^{240,241}. In patients who harbor a genetic mutation, surgical decision making is based on the mutation identified and associated hereditary cancer syndrome (e.g. Lynch Syndrome or Familial Adenomatous Polyposis). Risk of metachronous cancer as well as anticipated functional outcomes should be considered. For a first colonic cancer associated with Lynch Syndrome, segmental resection is preferable for lower risk pathogenic variants *MSH6* or *PMS2*, whilst

extended surgery is recommended for higher risk pathogenic variants *MLH2* or *MLH6*¹²³. Standard anterior resection or abdominoperineal resection depending on tumour location is recommended for rectal cancers.

The sensitivity of EOCRC to conventional chemotherapy is unknown as young patients account for a small proportion of clinical trials.

Current treatment algorithms do not differentiate between early and late onset disease. In practice however, several studies have shown young patients are more likely to receive adjuvant chemotherapy and more likely to receive multi-agent regimes (such as capecitabine and oxaliplatin (CAPOX), and fluorouracil, leucovorin and oxaliplatin (FOLFOX), at all stages compared to their older counterparts, despite no significant gain in survival^{113,114,135,242}. Furthermore, young patients are more likely to receive systemic therapy outside of National Comprehensive Cancer Network treatment guidelines in the absence of a definitive oncological benefit^{113,136,221,238}. Some patients with stage I or low-risk stage II colon cancer received adjuvant chemotherapy despite such treatment not being indicated. These data illustrate how young patients are at risk of overtreatment.

In this series, adjuvant chemotherapy was associated with a modest improvement in DFS among patients with stage III colon cancer. A larger sample size would be needed to demonstrate statistical significance. Interestingly, worse DFS was observed in patients with stage II disease who received adjuvant chemotherapy, suggesting the

presence of unfavorable histopathological features. Similarly, patients with stage III rectal cancer who received neoadjuvant therapy had worse DFS than those who did not. Evidently, an emphasis on alternative or adjunctive oncotherapeutic strategies such as total neoadjuvant therapy or immunotherapy with inhibitory checkpoint blockade should be explored to optimize survivorship^{67,218}.

Assessment of MSI status is an important screening tool for Lynch Syndrome (LS). LS, the most common hereditary cancer syndrome, is due to pathogenic constitutive variants in one of the mismatch repair proteins (*MLH2*, *MLH6*, *MSH2*, *PMS2*) and accounts for approximately 12-18% of EOCRCs^{131,134}. The estimated prevalence of hereditary cancer syndromes in EOCRC ranges between 5-35%, compared to 2-5% of CRCs overall¹²⁹⁻¹³¹. In the present study, MSI was identified 1 in 5 patients (1 in 4 colon cancers and 1 in 10 rectal cancers). A hereditary cancer syndrome was diagnosed in just over 1 in 3 patients with MSI colon cancer and 1 in 4 with MSI rectal cancer, highlighting the importance of reflective genetic testing in patients with EOCRC. The therapeutic implication of identifying MSI is the potential for immunotherapy with checkpoint blockade. Remarkable results have been observed in MSI CRC^{137,218,243}.

Limitations of this analysis include the retrospective nature, data heterogeneity, and variable treatment algorithms across the collaborative group. However, this study presents real-world data relating a disease that has been, until recently, relatively uncommon in

the young age group. Particularly pertinent findings are the proportion of patients with MSI and/or a definable hereditary cancer syndrome. The opportunities for molecular-targeted neoadjuvant and post-operative therapy represent an exciting challenge to the oncology community.

Chapter 5. Comparing disease-specific outcomes of patients with early age onset colon cancer according to microsatellite status

Abstract

5.1 Background

5.2 Methods

5.2.1 Study participants

5.2.2 Data collection

5.2.3 Study endpoints

5.2.4 Statistical analysis

5.3 Results

5.3.1 Baseline demographics

5.3.2 Pathological features

5.3.3 Molecular characteristics

5.3.4 Survival

5.3.5 Disease recurrence

5.3.6 Factors predictive of disease-specific outcomes

5.3.7 Comparison of sporadic MSI tumours and MSI tumours arising in the context of a hereditary cancer syndrome

5.4 Discussion

Abstract

Background: Tumours with microsatellite instability display distinct clinical and immunopathological characteristics. This study evaluated clinicopathological features and oncological outcomes according to microsatellite status in young patients with colon cancer.

Methods: Anonymised data from an international collaboration were analysed. Criteria for inclusion were patients young than 50 diagnosed with stage I-III colon cancer surgically resected with curative intent. Clinicopathological features and disease specific outcomes were compared according to microsatellite status.

Results: A total of 650 patients fulfilled the criteria for inclusion. Microsatellite instability (MSI) was identified in 170. The remaining 470 had microsatellite stable (MSS) tumours. MSI was associated with younger age at diagnosis (median age 40 vs 44, $p < 0.001$), a family history of colorectal cancer (19.5% vs 14.7%, $p = 0.001$) and tumours located in the proximal colon. The rates of pathological node positive disease were similar (45.9% vs 45.6%, $p = 1.000$).

A hereditary cancer syndrome was more common in patients with MSI (30.0% vs 5.0%, $p < 0.0001$). Five-year disease free survival rates in the MSI group were 95%, 92%, and 80% for stage I, II, and III, compared to 88%, 88%, and 65% in the MSS group ($p = 0.753, 0.487$, and 0.105 respectively).

Conclusion: In contrast to late-onset colon cancer, MSI in young adults was not associated with reduced likelihood of nodal positivity. Patients with MSI demonstrated better survival across all disease stages although the differences were not statistically significant.

5.1 Background

The incidence of colon cancer in adults aged less than 50 years is increasing worldwide^{8,9,102}. The clinical, pathological and molecular features of early age-onset colon cancer (EOCC) appear to differ somewhat to that of late-onset disease. Young patients typically present with advanced disease stage, and more frequently display unfavourable histopathological features such as poor differentiation, perineural invasion, venous invasion, and mucinous and/or signet cell morphology^{111,112,118}.

Histopathological analysis of the resected specimen and the Tumour, Node, Metastasis (TNM) staging system largely guides prognostication and therapeutic decision making (e.g. administration adjuvant chemotherapy) in patients with colon cancer. Patients with the same disease stage however can have very different clinical outcomes. This variation is likely related (at least in part) to differences in the molecular profile of the tumour. Microsatellite instability (MSI), due to defective DNA mismatch repair (MMR) which leads to the accumulation of errors in DNA replication, is a feature of 15% of CRCs^{25,30}. The defect may be sporadic or as a result of constitutive mutations in one of the MMR genes i.e. Lynch Syndrome¹⁷⁹. Current guidelines recommend that all CRC tumours should be tested for MSI either by immunohistochemistry or PCR-based methods.

MSI tumours display distinct clinical and pathological features. Often located in the proximal colon, they are less likely to metastasise to lymph nodes and distant organs and are associated with a robust intra-tumoral lymphocytic reaction^{178,182,244}. In comparison to MSS tumours, they exhibit better stage-adjusted survival, whilst their relative sensitivity to 5-fluorouracil-based chemotherapy remains controversial^{76,77,245,246}. These features however relate to all age CRCs. The clinicopathological characteristics of MSI cancers in early age onset disease are undefined. The aim of this study was to evaluate the clinicopathological features and oncological outcomes of non-metastatic colon cancers in patients diagnosed under the age of 50 years according to microsatellite status.

5.2 Methods

5.2.1 Study participants

A retrospective international multicentre observational study was performed to assess the clinicopathological features, molecular characteristics and disease specific outcomes of patients diagnosed with early age onset colon cancer. Inclusion criteria were adults aged between 18 and 49 years with a histologically confirmed diagnosis of non-metastatic colon cancer, undergoing surgery with curative intent, and known MSI status.

5.2.2 Data collection

Patients who fulfilled the inclusion criteria of the study were identified from the REACCT Collaborative database. Collected data included baseline patient demographics, clinical, stage, surgical, and treatment data, histopathological and molecular features, and cancer-specific as well as overall survival information. Clinical staging was according to the American Joint Committee on Cancer tumour node metastasis (TNM) staging system. Microscopically clear resection (R0) was defined by a tumour-free resection margin of at least 1 mm. Microsatellite instability was determined by polymerase chain reaction (PCR) or immunohistochemistry (IHC). Loss of mismatch repair (MMR) proteins *MLH1*, *PMS2*, *MSH2* or *MSH6* on IHC was classified as MSI. A hereditary cancer syndrome was defined as diagnosis of a constitutive pathogenic variant on germline testing.

5.2.3 Study endpoints

The primary outcome of interest was impact of MSI on oncological outcomes. The study endpoint was disease-specific survival.

5.2.4 Statistical Analysis

Continuous variables were described as mean ($\pm$ standard deviation) or median values (range) and compared by the Student t test or Mann-Whitney U test, depending on their distribution, Categorical variables were reported as percentages. Association of categorical variables was assessed using χ^2 test or Fisher exact test where appropriate. Survival statistics were calculated using the Kaplan-Meier method, and the log-rank test was used to assess differences in survival between groups.

Independent variables were entered into a multivariable binary logistic regression model. Variables that were found at univariable analysis to be significant, or a p-value <0.1 were entered into the multivariable model. A significance level of 0.05 was used for all analyses; reported p-values are 2-tailed. Data were analyzed using SPSS® software version 24.0 (IBM, Armonk, New York, USA).

5.3 Results

5.3.1 Baseline demographics

A total of 650 patients fulfilled the inclusion criteria. The median (range) age was 43 (18-49) years and 332 (51.1%) were male. Of those, 170 (26.2%) had tumours with defined MSI. The remaining 480 (73.8%) had MSS tumours. MSI was associated with younger age at diagnosis, a first degree relative with CRC, and right-sided lesions (caecum and ascending colon) but not with sex or body mass index. Demographics and clinical characteristics of the study population are summarized in *Table 10*.

Table 10. Comparison of demographics and clinicopathological data according to microsatellite status in patients with colonic cancer

	Overall N=650	MSI N=170	MSS N=480	p
Median age (range)	43(18-49)	40 (18-49)	44 (19-49)	<0.001
Male, N (%)	332 (51.1%)	111 (54.1%)	317 (50.1%)	0.176
Median BMI (range)	24.3 (13-58)	24.6 (13-47)	24.3 (16-58)	0.079
IBD	27 (4.2%)	5 (2.9%)	22 (4.6%)	1.000
FDR with CRC	127 (19.6%)	52 (30.7%)	75 (15.6%)	0.001
Tumour site				
Rectosigmoid junction	98 (15.1%)	10 (5.9%)	88 (18.3%)	<0.001
Sigmoid colon	200 (30.8%)	41 (23.9%)	159 (33.1%)	0.015
Descending colon	50 (7.7%)	12 (7.3%)	38 (7.9%)	0.881
Splenic flexure	64 (9.8%)	21 (12.7%)	43 (9.0%)	0.144
Transverse colon	50 (7.7%)	13 (7.8%)	37 (7.9%)	1.000
Hepatic flexure	16 (2.5%)	7 (4.4%)	9 (1.9%)	0.080
Ascending colon	90 (13.8%)	32 (19.0%)	58 (12.1%)	0.019
Caecum	82 (12.6%)	34 (20.0%)	48 (10.0%)	0.001
Synchronous tumour	6 (0.9%)	2 (1.2%)	4 (0.8%)	0.456
pStage				
I	118 (18.2%)	27 (15.8%)	91 (19.0%)	0.418
II	235 (36.2%)	65 (38.2%)	170 (35.4%)	0.517
III	297 (45.7%)	78 (45.9%)	219 (45.6%)	1.000
Adjuvant CT	408 (62.8%)	102 (60.0%)	306 (63.8%)	0.169

IBD = inflammatory bowel disease. FDR = first degree relative. pStage = pathological stage according to AJCC. CT = chemotherapy.

5.3.2 Pathological features

The proportion of patients with pathological stage I-III was similar in both groups. MSI tumours were more likely to be poorly or undifferentiated (28.1% vs 21.2%, $p=0.026$), and to display signet ring morphology (10.9% vs 4.4%, $p=0.013$). Lymphovascular (38.0% vs 47.5%, $p=0.027$) and extramural venous invasion (31.7% vs 44.9%, $p=0.008$) were less common in the MSI group. There was no difference in the rate of tumour budding between groups (20.3% vs 20.5%, $p=1.000$).

5.3.3 Molecular characteristics

BRAF and KRAS mutations were more common in patients with MSI tumours (22.5% vs 6.9%, $p < 0.001$, 40.0% vs 24.2%, $p = 0.006$). In the MSI group, 30.0% of patients ($n=51$) were diagnosed with a hereditary cancer syndrome compared to 5.0% of patients ($n= 24$) with MSS tumours (HR 8.142, 95% CI 5.12, 12.95, $p = <0.0001$). At the time of data collection, genetic testing had been performed in 62%.

5.3.4 Survival

Overall median follow-up was 48 months (1-221). The 5-year OS rates in the MSI group were 100%, 97%, and 86% for stage I, II, and III respectively, compared to 98%, 95%, and 77% in the MSS group. The 5-year DFS rates in the MSI group were 95%, 92%, and 80% for stage I, II, and III respectively. Corresponding values were 88%, 88%, and 65% in the MSS group (*Figures 7-12*). Whilst patients with MSI tumours demonstrated better survival across all disease stages, the differences were not statistically different. The greatest survival benefit with MSI was observed in stage 3 disease (80% vs 65%).

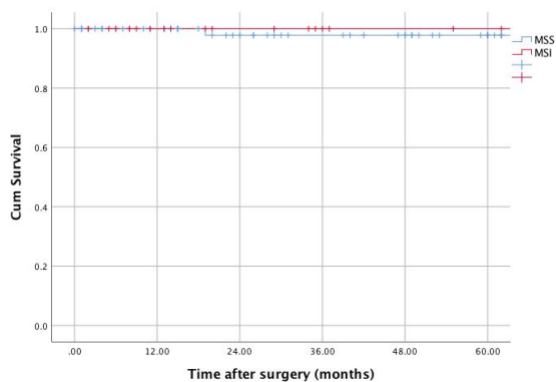


Figure 7. Kaplan-Meier curve of overall survival for patients with Stage I colon cancer according to microsatellite status. Overall survival did not differ significantly between patients with MSI and MSS tumours (Log rank test, $p = 0.595$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	21	15	11	8	6	5
MSS	67	53	42	34	29	20

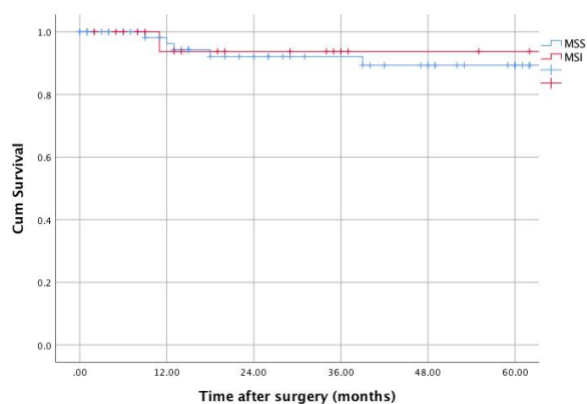


Figure 8. Kaplan-Meier curve of disease free survival for patients with Stage I colon cancer according to microsatellite status. Disease free survival did not differ significantly between patients with MSI and MSS tumours (Log rank test, $p = 0.753$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	21	15	11	8	6	5
MSS	67	49	37	31	25	18

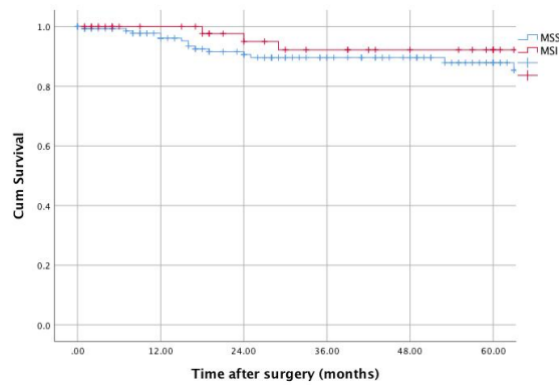


Figure 9. Kaplan-Meier curve of overall survival for patients with Stage II colon cancer according to microsatellite status. Overall survival did not differ significantly between patients with MSI and MSS tumours (Log rank test, $p = 0.241$)

No. at risk	0m	12m	24m	36m	48m	60m
MSI	57	45	38	33	29	25
MSS	147	119	101	80	67	48

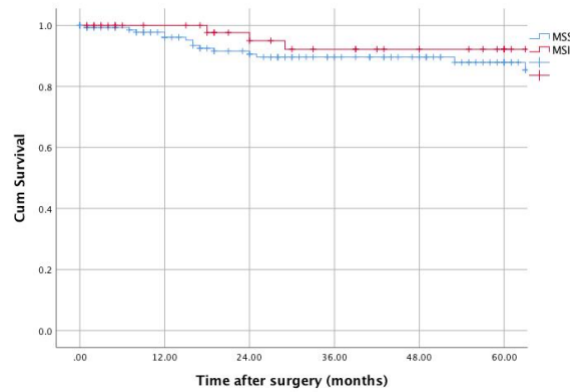


Figure 10. Kaplan-Meier curve of disease free survival for patients with Stage II colon cancer according to microsatellite status. Disease free survival did not differ significantly between patients with MSI and MSS tumours (Log rank test, $p = 0.487$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	57	45	38	32	28	24
MSS	147	118	94	73	60	42

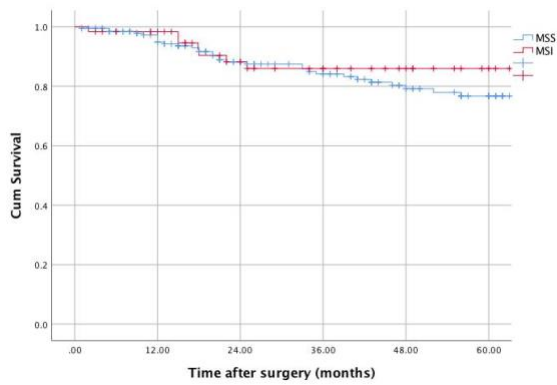


Figure 11. Kaplan-Meier curve of overall survival for patients with Stage III colon cancer according to microsatellite status. Patients with MSI had better OS but the differences were not statistically significant (Log rank test, $p = 0.261$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	73	66	55	36	27	21
MSS	196	163	121	100	72	56

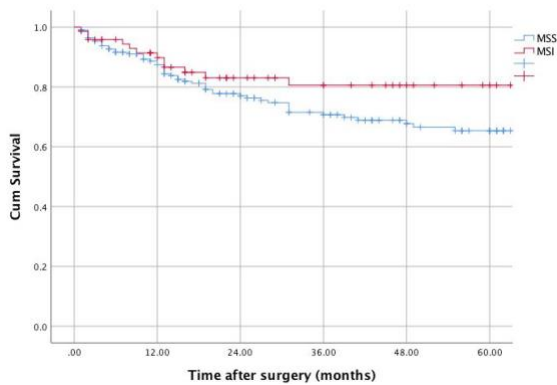


Figure 12. Kaplan-Meier curve of disease free survival for patients with Stage III colon cancer according to microsatellite status. Patients with MSI had better DFS but the differences were not statistically significant (Log rank test, $p = 0.105$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	73	58	39	33	26	21
MSS	196	148	107	86	61	49

5.3.5 Disease recurrence

In the MSI group, 17 patients (10.0%) developed disease recurrence compared with 71 (14.8%) in the MSS group ($p = 0.053$).

Locoregional recurrence occurred in 5 patients (2.9%) with MSI tumours and in 18 patients (3.8%) with MSS tumours ($p = 0.638$).

Distant disease developed in 16 (9.4%) and 76 (15.8%) respectively ($p = 0.083$). The median time to recurrence was 12 (1-84) months after surgery among patients with MSI tumours, and 13 (1-63) months in those with MSS tumours ($p = 0.480$).

5.3.6 Factors predictive of disease-specific outcomes

On univariable analysis, in the MSI group, lymphovascular, and extramural venous invasion were associated with worse DFS (*Table 11*). Only extramural venous invasion was significant on multivariable analysis (HR 7.81, 95% CI 1.89, 32.22, $p = 0.004$). On univariable analysis, in the MSS group, R0 resection was significantly associated with better DFS, whilst signet ring morphology, lymphovascular, extramural, and perineural invasion, were associated with worse DFS. On multivariable analysis, only lymphovascular invasion (HR 2.294, 95% CI 1.36, 3.94, $p = 0.003$) was significantly associated with worse DFS (*Tables 11 & 12*).

Table 11. Univariable Logistic Regression of Factors Predicting Disease**Free Survival for MSI cancers**

Variable	HR	95% CI	<i>p</i>
Age (years)	0.997	.05, 1.04	0.888
Poor differentiation	1.185	.79, 1.79	0.418
Tumour Budding	1.022	.19, 5.47	0.980
Signet Ring	2.933	.68, 12.65	0.149
Mucin $\geq$ 50%	2.209	.74, 6.56	0.153
LVI	3.357	1.22, 9.25	0.019
EMVI	4.494	1.76, 12.55	0.002
Perineural invasion	2.682	.95, 7.54	0.061
R0 resection	0.143	.01, 2.77	0.198
Node positive (pN)	1.333	.50, 3.55	0.565

LVI = lymphovascular invasion. EMVI = extramural venous invasion. pN+ = pathological node positive

Table 12. Univariable Logistic Regression of Factors Predicting Disease Free Survival for MSS cancers

Variable	HR	95% CI	<i>p</i>
Age (years)	0.995	.97, 1.02	0.747
Poor differentiation	1.194	.98, 1.46	0.082
Tumour Budding	1.133	.57, 2.19	0.711
Signet Ring	2.780	1.17, 6.61	0.021
Mucin $\geq$ 50%	1.718	.98, 3.02	0.060
LVI	2.478	1.67, 3.72	<0.001
EMVI	2.088	1.38, 3.17	0.001
Perineural invasion	2.302	1.52, 3.50	<0.001
R0 resection	0.510	.29, .89	0.018
Node positive (pN+)	1.171	.79, 1.74	0.437

LVI = lymphovascular invasion. EMVI = extramural venous invasion. pN+ = pathological node positive

5.3.7 Comparison of sporadic MSI tumours and MSI tumours arising in the context of a hereditary cancer syndrome

Sub-group analysis of MSI tumours was performed comparing patients with sporadic tumours to those with tumours and a confirmed genetic predisposition. Of the 170 MSI tumours, 44 had confirmed sporadic tumours whilst 61 patients had a confirmed hereditary cancer syndrome. There were no significant differences in baseline demographics, clinical characteristics or pathological features. Patients with a hereditary cancer syndrome were more likely to have a first degree relative with CRC (35.5% vs 7.5%, $p = 0.001$). No significant differences in overall and disease specific survival were observed. The overall survival rate at 1, 3 and 5 years was 100%, 93%

and 93% for those with a hereditary cancer syndrome compared to 97%, 91% and 91% for those with sporadic disease ($p = 0.643$). The disease-free survival rate at 1, 3 and 5 years was 98%, 90%, and 90% for patients with a hereditary cancer syndrome compared to 97%, 86%, and 86% for those with sporadic disease ($p = 0.792$.) Sub-analysis on the basis of disease stage, did not reveal significant differences in disease specific survival between the groups.

5.4 Discussion

Understanding the biological and pathological mechanisms that underpin tumour development is important to optimise outcomes and guide therapeutic decision making. In the present study, MSI was associated with a family history of CRC, and lesions in the proximal colon. Unlike in older age groups, MSI was not associated with a female preponderance. A particularly noteworthy result from this data, is the equal rate of nodal positivity in young patients with MSI and MSS colon cancer. This is in contrast to the findings of studies evaluating all age MSI colon cancers, where MSI was associated with a lower likelihood of lymph node involvement²⁴⁴.

In keeping with previous reports of all age cancers, poor differentiation and signet ring morphology were associated with MSI. The presence of tumour budding (TB) however, a biomarker of metastatic potential and negative prognostic indicator, was similar in both MSI and MSS tumours^{84,247}. Several studies of older patients with CRC have found that TB is less common in MSI cancers^{83,248}. The lower rate of TB in all age MSI cancers may in part explain why these tumours are less likely to metastasise to lymph nodes and distant organs. Larger prospective studies (with standardized assessment of TB) however, would be needed to confirm these findings.

Patients with MSI cancers have better stage-adjusted survival than those with MSS cancers^{32,249-252}. MSI has been demonstrated as a positive prognostic indicator in stage II colon cancer but its value as a

prognostic marker stage III disease is less clear. Several studies observed no difference in survival in patients with node positive disease^{244,253}. In the present study, patients with MSI had better disease specific outcomes in all stages, although the differences observed were not statistically significant. Large numbers would be required for statistical power. The differences in survival rates however, are likely to be of clinical significance. In particular for patients with stage III disease, in whom 5-year DFS was 80% in the MSI group and 65% in the MSS group. This survival advantage of MSI in node positive disease may be related to the distinct immunological features of these tumours. Whether and how lymph nodes influence the development of distant metastases is unclear. Murine studies in melanoma have shown that tumour cells in the lymph nodes acquire the ability to resist T-cell mediated cytotoxicity, thereby evoking immune tolerance that enables spread to distant organs²⁵⁴. Tumours with MSI demonstrate a strong peritumoral lymphocytic reaction in comparison to MSS tumours. It can be hypothesized that a similar immune response also occurs in the lymph nodes, potentially limiting the development of distant metastases and thus improving DFS.

Interestingly, poor prognostic pathological features (poor differentiation, mucinous histology) in all age MSI cancers have historically not been associated with adverse outcome. Despite comparable rates of tumour budding in the present study, the MSI group demonstrated better disease-specific survival.

Importantly, MSI serves as a screening tool for genetic testing for Lynch Syndrome which accounts for one-third of CRC cases under the age of 35^{131,133}. The prevalence estimates of hereditary cancer syndromes among patients with EOCRC ranges between 5 and 35% compared to 2-5% of CRCs overall¹²⁹⁻¹³¹. In the present series, pathogenic constitutive variants more common in the MSI group (1 in 3 patients) than the MSS group (1 in 20 patients). The clinical implications of identification of genetic predisposition include increased cancer surveillance, the potential of prophylactic risk-reducing surgery, and testing of at-risk relatives. Clinical trials investigating preventative vaccines for patients with Lynch Syndrome are ongoing. It is hoped that vaccination with the mutated proteins (neoantigens) will prepare the immune system in advance to reject tumour cells if they develop²⁵⁵.

Limitations of this study include its retrospective nature, lack of complete dataset for the entire study group, and heterogeneity in treatment across the collaborative group. Despite a large cohort of patients, it is possible that the findings of this study are due to a type II statistical error and lack of statistical power. Nonetheless, data specific to early age onset disease are lacking and this study presents large volume real-world data. Importantly, early onset MSI tumours display several differences to late-onset MSI tumours. In contrast to older age groups, MSI in young patients is not associated with female preponderance, and is associated with equal rates of nodal positivity and tumour budding as MSS tumours.

Chapter 6. The impact of MSI on treatment response and oncological outcomes in patients with early age onset rectal cancer

Abstract

6.1 Background

6.2 Methods

6.2.1 Study participants

6.2.2 Data collection

6.2.3 Study endpoints

6.2.4 Statistical analysis

6.3 Results

6.3.1 Baseline demographics

6.3.2 Pathological features

6.3.3 Molecular characteristics

6.3.4 Oncological outcomes

6.3.5 Factors predictive of disease-specific outcomes

6.4 Discussion

Abstract

Background: The molecular profile of colorectal cancer has important prognostic and therapeutic implications. This study evaluated the impact of microsatellite status on treatment response and disease specific outcomes in young adults with rectal cancer.

Methods: Anonymised data from an international collaboration were analysed. Criteria for inclusion were patients younger than 50 diagnosed with stage I-III rectal cancer surgically resected with curative intent. Clinicopathological features and disease specific outcomes were compared according to microsatellite status.

Results: A total of 400 patients fulfilled the criteria for inclusion of whom 50 had tumours with defined microsatellite instability (MSI). The remaining 350 had microsatellite stable (MSS) tumours. Clinical stage was similar in both groups. MSI was associated with a first degree relative with colorectal cancer (20% vs 8.9%, $p = 0.009$). A higher proportion of patients with MSI achieved a complete pathological response (32.3% vs 15.7%, $p = 0.044$). Patients with MSI were less likely to have pathological node positive disease (22.0% vs 41.7%, $p = 0.008$). Tumours with MSI were more likely to occur in the context of a hereditary cancer syndrome (30.0% vs 3.1%, $p < 0.0001$). Five-year disease free survival rates in the MSI group were 87% compared to 66% in the MSS group.

Conclusion: MSI in young adults with rectal cancer was associated with an increased pCR rate which opens the possibility of organ preservation in this group. Patients with MSI were also less likely to be node positive and demonstrated better disease-free survival.

6.1 Background

Deciphering the underpinning biomolecular processes in colorectal cancer (CRC) has been a major research focus of the past few decades. Several forms of genetic instability have been described, of which chromosomal instability (CIN) is the most common accounting for 85% of cases. The remaining 15% display microsatellite instability (MSI)³⁰.

MSI is a well-defined feature of defective DNA mismatch repair (MMR), and may occur due to sporadic epigenetic silencing of the *MLH1* gene or constitutive mutations in one of the MMR genes (i.e. Lynch Syndrome)¹³². Reflex testing of MMR or MSI status is now recommended for all patients diagnosed with CRC regardless of age or family history. In addition to being an important screening tool for Lynch Syndrome, MSI status also provides valuable prognostic information as MSI is associated with improved disease-specific survival^{76,256}. The therapeutic implications of MSI are less clear. Controversy exists as to whether MSI confers a relative resistance to 5-fluorouracil (5-FU) chemotherapy, and the impact of MSI status on response to neoadjuvant chemoradiotherapy remains undefined^{77,245,257-259}.

One of the most notable epidemiological changes of the modern era, is the rising incidence of early onset rectal cancer (EORC), defined as diagnosis under 50 years^{8,9,113,260}. Whether the biological processes underpinning early-onset disease differ to that of late-onset is unclear.

Although similar key oncogenic pathways are implicated, some biomolecular differences have been observed which may have potential therapeutic implications^{103,119,120,234}. The majority of cases of EORC are sporadic and microsatellite stable, despite young age at diagnosis being a hallmark of genetic predisposition^{130,135}. As young patients have historically represented a small proportion of clinical trials, the impact of microsatellite status on treatment response and disease-specific outcomes in this patient group is unknown. The objective of this study was to evaluate the impact of microsatellite status on oncological outcomes in patients diagnosed with non-metastatic rectal cancer aged less than 50 years.

6.2 Methods

6.2.1 Study participants

A retrospective international multicentre observational cohort study to assess the clinicopathological features, molecular characteristics and disease specific outcomes of patients diagnosed with early age onset rectal cancer over a 20-year period (2000 to 2020) was performed. Inclusion criteria were adults aged between 18 and 49 years with a histologically confirmed diagnosis of rectal cancer, undergoing surgery with curative intent, and known MSI status. Rectal cancer was defined as tumour location within 15cm from the anal verge on rigid sigmoidoscopy. Patients with metastatic disease at diagnosis were excluded.

6.2.2 Data collection

Patients who fulfilled the inclusion criteria of the study were identified from the REACCT Collaborative database. All participating institutions were tertiary referral centres with expertise in CRC. The indication for and type of neoadjuvant therapy and adjuvant therapy, restaging, pathological assessment, and follow up were according to the local institutional protocols. A principle investigator in each institution was responsible for data collection. Ethical approval was obtained at institutional level. Anonymised data was entered into REDCap. Collected data included baseline patient demographics, clinical, stage, surgical, and treatment data, histopathological and molecular features, and cancer-specific as well as overall survival information. Clinical staging was according to the American Joint

Committee on Cancer tumour node metastasis (TNM) staging system. Microscopically clear resection (R0) was defined by a tumour-free resection margin of at least 1 mm. Microsatellite instability was determined by PCR or immunohistochemistry (IHC). Loss of mismatch repair (MMR) proteins *MLH1*, *PMS2*, *MSH2* or *MSH6* on IHC was classified as MSI. A hereditary cancer syndrome was defined as diagnosis of a constitutive pathogenic variant on germline testing.

6.2.3 Study endpoints

The primary outcome of interest was impact of MSI on oncological outcomes and treatment response.

6.2.4 Statistical Analysis

Continuous variables were described as mean ($\pm$ standard deviation) or median values (range) and compared by the Student t test or Mann-Whitney U test, depending on their distribution, Categorical variables were reported as percentages. Association of categorical variables was assessed using χ^2 test or Fisher exact test where appropriate. Survival statistics were calculated using the Kaplan-Meier method, and the log-rank test was used to assess differences in survival between groups. Independent variables were entered into a multivariable binary logistic regression model. Variables that were found at univariable analysis to be significant, or a p-value <0.1 were entered into the multivariable model. A significance level of 0.05 was used for all analyses; reported p-values are 2-tailed. Data were analyzed using SPSS® software version 24.0 (IBM, Armonk, New York, USA).

6.3 Results

6.3.1 Baseline demographics

A total of 400 patients diagnosed over a 20-year interval who fulfilled the inclusion criteria were included in the study. Of those 400 patients, 50 (12.5%) had tumours with defined MSI. The remaining 350 (87.5%) had microsatellite stable (MSS) tumours. Overall, the median (range) age was 43 (23-49) years and 204 (58.3%) were male. In the MSI group, women accounted for 58%. MSI was associated with a first degree relative with CRC. No difference in clinical stage was observed between the two groups. Summarised in *Table 13*.

Table 13. Comparison of demographics and clinicopathological data according to microsatellite status in patients with rectal cancer

	Overall N=400	MSI N=50	MSS N=350	p
Median age (range)	43 (23-49)	39 (26-49)	48 (23-49)	0.293
Male, N (%)	204 (51.0%)	21 (42%)	183 (52.3%)	0.137
FDR with CRC	41 (10.3%)	10 (20%)	31 (8.9%)	0.009
cStage I-II	97 (24.3%)	9 (18.0%)	88 (25.1%)	0.091
III	208 (52.0%)	28 (56%)	180 (51.4%)	0.091
Unknown	48 (23.8%)	13 (26.0%)	82 (23.4%)	
Neoadjuvant CRT	248 (62.0%)	31(62.0%)	217 (62.0%)	1.000
(y)pStage				
I	117 (29.3%)	14 (28.0%)	103 (29.4%)	1.000
II	126 (31.5%)	25 (50.0%)	101 (28.9%)	0.005
III	157 (39.2%)	11 (22.0%)	146 (41.7%)	0.008
Pathology				
pCR	44 (17.7%)	10 (32.3%)	34 (15.7%)	0.044
R0 resection	372 (93.0%)	47 (94.0%)	325 (92.9%)	1.000
HCS	26 (6.5%)	15 (30.0%)	11 (3.1%)	<0.0001
Adjuvant CT	249 (62.3%)	28 (56.0%)	221 (63.1%)	0.189

FDR = first degree relative. cStage = clinical stage according to AJCC. CRT = chemoradiotherapy. y = post neoadjuvant therapy. pStage = pathological stage according to AJCC. pCR = pathological complete response. HCS = hereditary cancer syndrome. CT = chemotherapy

6.3.2 Pathological features

There were no significant differences in differentiation, or lymphovascular, extramural venous or perineural invasion between the two groups. A higher proportion of patients with MSI achieved a complete pathologic response (pCR) (32.3% vs 15.7%, $p = 0.044$). Patients in the MSI group were less likely to have pathological node positive disease (22.0% vs 41.7%, $p = 0.008$). These data are summarised in Table 14.

Table 14. Comparison of pathological features according to microsatellite status in patients with rectal cancer

	Overall	MSI	MSS	p
Differentiation				
Well	95 (27.6%)	13 (30.2%)	82 (27.2%)	0.667
Moderate	186 (54.1%)	24 (55.8%)	162 (53.8%)	
Poor/undifferentiated	64 (18.6%)	6 (14.0%)	58 (19.2%)	
LVI	125 (33.9%)	16 (38.1%)	109 (33.3%)	0.612
EMVI	95 (29.1%)	10 (25.0%)	85 (29.7%)	0.582
Perineural invasion	54 (15.5%)	6 (12.8%)	58 (19.2%)	0.243
Tumour regression grade				
TRG 0 (ypT0N0)	44 (17.7%)	10 (32.3%)	34 (15.7%)	0.044
TRG 1	19 (7.7%)	1 (3.2%)	18 (8.3%)	
TRG 2	109 (44.0%)	9 (29.0%)	100 (46.1%)	
TRG 3	62 (25%)	11(35.5%)	51 (23.5%)	
Unknown	14 (5.6%)	0	14 (6.5%)	

*Percentages are calculated from the total known data. LVI = lymphovascular invasion. EMVI = extramural venous invasion. TRG = tumour regression grade.

6.3.3 Molecular characteristics

Patients with MSI tumours were more likely to have a genetic predisposition. A hereditary cancer syndrome was diagnosed in 30.0% of patients (n=15) with MSI tumours compared to 3.1% of patients (n=11) with MSS tumours (HR 13.21, 95% CI 5.63-30.97, $p < 0.0001$). At the time of data collection, only 72.0% (n=36) of the MSI group and 65.7% (n=230) of the MSS group had undergone genetic testing.

6.3.4 Oncological outcomes

Survival

Survival data were available for 392 patients (98%). Overall median follow-up was 35 months (1-197). The median overall survival in the

MSI group was 58 months (1-197), with 1-, 3- and 5-year overall survival rates of 100%, 95% and 89% respectively. Corresponding values in the MSS group were 32 months (1-158) and 96%, 90% and 84% (*Figure 13*). In the MSI group, the median disease-free survival was 57 months (1-197) compared to 23 months (1-158) in the MSS group. In patients with MSI, the disease-free survival rate at 1, 3 and 5 years was 98%, 90% and 87%, compared with 89%, 72% and 66% among those with MSS tumours (*Figure 14*). On sub-analysis based on pathological stage, survival was better in the MSI group for stage I, II and III disease, however the differences were not statistically significant (*Figure 15*).

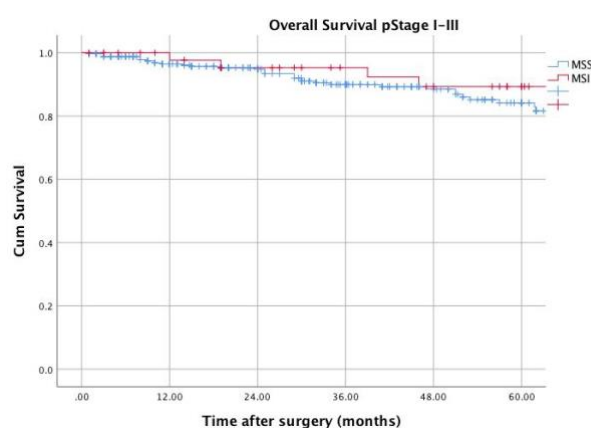


Figure 13. Kaplan-Meier curve of overall survival for patients with Stage I-III rectal cancer according to microsatellite status. Overall survival did not differ significantly between patients with MSI and MSS tumours (Log rank test, $p = 0.323$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	50	43	38	32	29	3
MSS	342	272	211	160	115	80

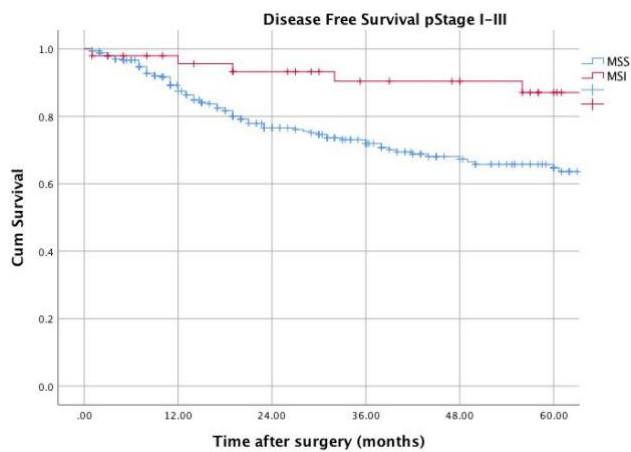
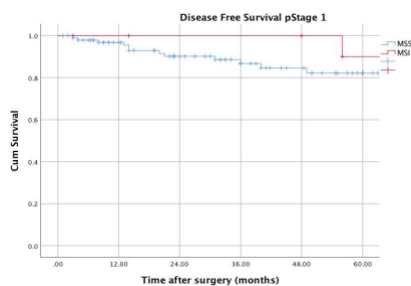


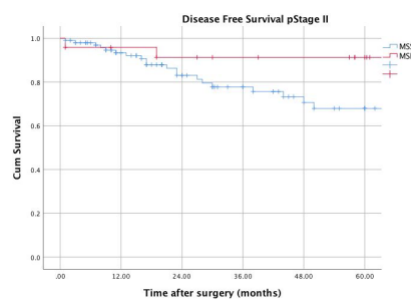
Figure 14. Kaplan-Meier curve of disease free survival for patients with Stage I-III rectal cancer according to microsatellite status. Patients with MSI had significantly better DFS (Log rank test, $p = 0.007$).

No. at risk	0m	12m	24m	36m	48m	60m
MSI	50	42	37	31	29	22
MSS	342	247	166	128	87	66



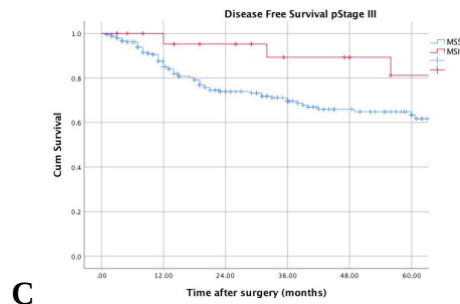
A

No. at risk	0m	12m	24m	36m	48m	60m
MSI	14	13	12	12	12	8
MSS	100	80	61	48	36	25



B

No. at risk	0m	12m	24m	36m	48m	60m
MSI	24	21	19	17	16	13
MSS	99	75	50	39	28	22



No. at risk	0m	12m	24m	36m	48m	60m
MSI	11	8	6	2	1	1
MSS	143	93	56	43	25	20

Figure 15. Kaplan-Meier curve of disease free survival for patients with Stage I (A), II (B) and III (C) rectal cancer according to microsatellite status. Patients with MSI had better survival across all disease stages however, the differences were not statistically significant (Log rank test, $p = 0.520$, 0.056 , and 0.264 respectively).

Disease recurrence

In the MSI group, no patient developed locoregional disease recurrence compared with 24 patients (6.9%) in the MSS group ($p = 0.159$). Five patients (10%) with MSI developed metastatic disease compared with 72 (20.6%) in the MSS group ($p = 0.084$).

6.3.5 Factors predictive of disease-specific outcomes

No variable was significantly associated with disease recurrence in the MSI group on univariable analysis. In univariable analysis, in the MSS group, lymphovascular, extramural, and perineural invasion, non-pCR, and node positivity were significantly associated with worse DFS. In multivariable analysis, only lymphovascular invasion (HR 2.831, 95% CI 1.09, 7.31, $p = 0.032$) and adjuvant chemotherapy (HR

4.893, 95% CI 1.29, 18.63, $p = 0.02$) were significantly associated with disease recurrence. (Tables 15 & 16)

Table 15. Univariable Logistic Regression of Factors Predicting Disease Recurrence for MSI rectal cancers

Variable	HR	95% CI	<i>p</i>
Age (years)	1.006	.89, 1.13	0.923
Male sex	3.176	.52, 19.27	0.209
Neoadjuvant CRT	1.185	.19, 7.22	0.854
LVI	1.770	.31, 10.07	0.520
EMVI	3.857	.64, 23.41	0.142
Perineural invasion	4.625	.64, 33.71	0.130
R0 resection	0.238	.02, 3.12	0.274
TRG 3	1.889	.23, 15.74	0.557
Node positive (pN)	1.944	.31, 12.35	0.481
Adjuvant CT	1.330	.22, 8.16	0.756

CRT = chemoradiotherapy. LVI = lymphovascular invasion. EMVI = extramural venous invasion. TRG = tumour regression grade. CT = chemotherapy

Table 16. Univariable Logistic Regression of Factors Predicting Disease Recurrence for MSS rectal cancers

Variable	HR	95% CI	<i>p</i>
Age (years)	0.994	.96, 1.03	0.773
Male sex	1.044	.64, 1.69	0.860
Neoadjuvant CRT	1.786	1.06, 3.02	0.031
LVI	2.771	1.64, 4.68	<0.001
EMVI	3.528	1.97, 6.31	<0.001
Perineural invasion	3.290	1.86, 5.81	<0.001
R0 resection	0.336	.15, .77	0.010
TRG 3	1.810	1.25, 2.61	0.002
Node positive (pN)	2.965	1.79, 4.89	<0.001
Adjuvant CT	2.175	1.24, 3.81	0.006

CRT = chemoradiotherapy. LVI = lymphovascular invasion. EMVI = extramural venous invasion. TRG = tumour regression grade. CT = chemotherapy

6.4 Discussion

The molecular stratification of patients with CRC has been facilitated by increased understanding of the biomolecular processes that underly tumour development. Assessment of MSI status is the most commonly used molecular classification system in clinical practice.

Unsurprisingly, tumours that arise from different oncogenic pathways differ clinically. In this study of 400 patients with early age onset rectal cancer, 12.5% of patients had tumours with MSI. MSI was associated with reduced likelihood of nodal positivity, increased rate of pCR and improved disease-specific survival. Patients with MSI tumours were also more likely to be diagnosed with a hereditary cancer syndrome.

EOCRC has increased worldwide over the past four decades^{9,100,101}. The global increase is predominantly driven by a rise in the rate of rectal tumours⁸. Historically, males have accounted for a greater proportion of patients with rectal cancer than females. A nationwide Swedish registry-based study however, reported a male-to-female incidence rate ratio of 1.07 in adults aged 18-49, compared to 1.71 among those aged greater than 49²⁶¹. Generally, MSI is more frequently identified in females, particularly in patients with tumours in the caecum and ascending colon²⁵³. In the present study, although there were more males overall, the majority (58%) of patients in the MSI group were women. The impact of female sex on risk of early age rectal cancer or presence of MSI remains to be defined.

The role of molecular profile in therapeutic decision making continues to be defined. Neoadjuvant chemoradiotherapy is standard of care for locally advanced rectal cancer. Pathological response however varies considerably. Achieving a pCR is associated with low locoregional recurrence rates and excellent long-term outcomes^{62,262}. In the present study, an increased pCR rate was observed in patients with MSI rectal cancer. This opens the possibility of organ preservation in this specific group. As disease-free survival with a pCR is excellent, avoiding surgery may be an appropriate option in some of these patients²⁶³.

A ‘watch and wait’ strategy has been developed for patients who achieve a complete clinical response to nCRT in an effort to avoid unnecessary surgery. Select patients undergo strict surveillance with salvage surgery reserved for cases of local regrowth or recurrence. Clinicians may be hesitant to adopt an organ preservation in young patients than older patients. Concerns exist surrounding the oncological safety of this approach where life expectancy is longer and there is potential greater loss of life years. Promising data from the International Watch and Wait Database (IWWD), have shown that young patients have comparable disease-free survival as well as rates of local regrowth (22.1% vs 23.3%) and distant metastases (9.5% vs 8.9%) to that of older patients²⁶⁴. When patients have a sustained cCR for 3 years, the probability of developing local regrowth or distant metastases is less than 3%²⁶⁵.

In general, young adults with rectal cancer are otherwise healthy, and socioeconomic, psychosocial and quality of life factors are important. The presence of a stoma can have significantly negative impact on body image and confidence. Patients managed by a 'watch and wait' strategy have a significantly better 3-year colostomy-free survival than those who have immediate surgery and are more likely to avoid a permanent stoma²⁶⁶.

Patient preference and shared-decision making are important factors in the management of EORC. Some patients may be willing to accept the higher oncological uncertainty associated with organ preservation, whilst others may prefer the relative certainty of proceeding with surgery. Studies have shown that 83% of patients with rectal cancer would consider a 'watch and wait strategy' if they achieved a cCR, and 94% would accept a local regrowth risk of 25%^{267,268}. Avoidance of a permanent stoma in particular, was a priority. When patient and physician preferences were compared, patients were willing to accept a 20% absolute increase in local regrowth and a 20% absolute decrease in overall survival with non-operative management²⁶⁸. Physicians however, were only willing to accept a 5% absolute increase in local regrowth and a 5% absolute decrease in overall survival.

The data acquired from discrete choice experimental data in patients with pCR following chemoradiotherapy for oesophageal cancer, may

provide a useful platform from which to explore similar concepts in patients with EORC. Patients were prepared to give up life years in order to avoid the potentially disabling symptoms due to the anatomical, physiological and social impact of major surgery²⁶⁹. In particular, the negative impact of poor genitourinary and lower gastrointestinal function (i.e. low anterior resection syndrome) that may occur as a result of major pelvic surgery may be avoided with an organ preserving approach^{73,74}. However, there are negative consequences to neoadjuvant chemoradiotherapy which include (but are not limited to) diminished fertility, pelvic fractures, and neuropathy²⁷⁰⁻²⁷².

Young patients with rectal cancer demonstrate similar disease-specific survival to their older counterparts (despite receiving more neoadjuvant and adjuvant treatment)¹³⁵. Oncological outcomes according to MSI status specifically are limited. In the present study, patients with MSI demonstrated better disease-specific survival independent of disease stage. The differences were not statistically significant. Statistical analyses in relatively small cohorts however, must be interpreted with caution to avoid misleading results or inaccurate conclusions. Larger numbers would be required to achieve statistical power. Nonetheless, the absolute difference of 21% between 5-year disease-free survival (MSI vs MSS; 87% vs 66%) is certainly clinically significant. As expected, overall survival did not differ with between groups.

MSI status is an important screening tool for genetic cancer predisposition e.g. Lynch Syndrome. In the present study, 6.5% of patients overall were diagnosed with a genetic predisposition. These data, in keeping with other series, suggest that although a hereditary cancer syndrome is more likely in young adults with rectal cancer, a diagnosis is still infrequent¹³⁵. Patients with MSI however were 10 times more likely to be diagnosed with an inherited susceptibility. Almost 1 in 3 patients with MSI had a genetic predisposition (MSI vs MSS; 30.0% vs 3.1%) highlighting the importance of reflective genetic testing in this group. As the full spectrum of genes implicated in EOCRC is unknown however, it is possible that a proportion of patients with sporadic disease actually harbour mutations not yet identified^{130,131}. Advances in next-generation sequencing with multigene panel testing will unveil this spectrum.

Limitations of this study include its retrospective nature, lack of complete dataset for the entire study group, and heterogeneity in treatment across the collaborative group. Chemo/radiotherapy strategies have evolved over the study period of 20 years. Furthermore, the number of patients for whom survival data were collected was too small to analyse oncological outcomes on the basis of disease stage. Nonetheless, this study represents real-world data and provides a useful platform for future research. Neoadjuvant therapy should be a focus of modern trials in this patient group. Total

neoadjuvant therapy, which is associated with improved chemotherapy compliance and superior pCR rates, may represent an attractive strategy^{67,273}. Furthermore, there is potential for immunotherapy (in the form of checkpoint inhibitors) to be incorporated into the neoadjuvant treatment paradigm, in particular for patients with MSI tumours. The NICHE study demonstrated remarkable response to neoadjuvant checkpoint blockade among patients with non-metastatic colon cancer²¹⁸.

Chapter 7. Uncoupling of tumour-infiltrating lymphocyte checkpoint expression and microsatellite instability status in colorectal cancer

Abstract

7.1 Background

7.2 Methods

7.2.1 Study population

7.2.2 Pathological analysis

7.2.3 Immunophenotyping

7.2.4 Statistical analysis

7.3 Results

7.3.1 Baseline demographics and clinical data

7.3.2 Microsatellite instability status

7.3.3 Inhibitory checkpoint expression

7.3.4 Functional analysis of TILs

7.3.5 Association between PD-1 expression and clinicopathological parameters

7.4 Discussion

Abstract

Background: The immune microenvironment of colorectal cancer is complex and heterogeneous. Modern treatment strategies in cancer immunology have focused on inhibitory checkpoint blockade with variable clinical efficacy observed. Tumour biology, in particular microsatellite status, has been utilised as a predictive biomarker of response. The aim of this study was to evaluate inhibitory checkpoint expression on tumour-infiltrating lymphocytes in colorectal cancer according to microsatellite status.

Methods: A consecutive series of patients with histologically confirmed, non-metastatic, treatment naïve colorectal cancer undergoing surgery with curative intent were studied. Baseline demographic and clinicopathological data were recorded. Immunophenotyping was performed by flow cytometry and single cell RNA sequencing.

Results: Of the 20 patients included, the median (range) age was 73 (41-87), and 60% were male. The majority of tumours (95.2%) were located in the colon. Defined microsatellite instability was identified in 6 (30.0%) patients. Flow cytometry analysis found that expression of the inhibitory markers PD-1 and TIGIT were significantly increased on CD3⁺ cells (T cells) in the tumour compared to uninvolved healthy colonic tissue ($p=0.0036$, $p=0.0026$). PD-1 expression was significantly increased in tumours with microsatellite instability (MSI) compared to tumours that were microsatellite stable (MSS) ($p=0.040$), however one in four MSS tumours had levels of PD-1 expression comparable to the MSI group. TIGIT expression did

not differ between groups. Functional status assessing intra-tumoral CD3⁺ cell production of IFN γ , IL-17a, and amphiregulin did not differ significantly between groups.

Conclusion: Inhibitory checkpoint expression varies significantly among patients with the same microsatellite status. Microsatellite status alone may not accurately predict response to checkpoint blockade therapy.

7.1 Background

Although complete surgical excision is central to curative treatment of colorectal cancer (CRC), improved oncological outcomes have been achieved by the addition of systemic neoadjuvant and adjuvant therapy^{47,48,50}. Immunotherapy, through reinvigorating the patients' own immune response, or by allogenic or autologous immune cell adoptive therapy, has emerged as an alternative therapeutic strategy to improve disease control.

The immune microenvironment of cancer is heterogeneous. The diversity of immune infiltration has led to the increasingly used nomenclature of '*hot*' and '*cold*' tumours where '*hot*' tumours are those characterised by high T-cell infiltration while '*cold*' tumours demonstrate an absence of or poor T-cell infiltration¹⁴⁴. There is now strong data demonstrating that intra-tumoral immune landscape is an important predictor of disease-related outcome¹⁴³. Dense T-cell infiltration in CRC is associated with improved disease-specific survival and is prognostically superior to the standard TNM staging classification¹⁴⁵. Despite this data, incorporation of individual host immune response has not yet translated into routine clinical practice, in part due to uncertainty surrounding therapeutic implications.

Intra-tumoral immune response is influenced by tumour biology. DNA mismatch repair status is an established biomarker, dichotomizing CRC into mismatch repair-proficient (pMMR) and mismatch repair-deficient (dMMR). Defective DNA mismatch repair results in microsatellite

instability (MSI), where a high frequency of replication errors results in the accumulation of deletion/insertion mutations at coding microsatellites¹⁷⁸. These frame shift mutations inactivate tumour suppressor genes leading to tumorigenesis²⁶. Approximately 15% of CRCs develop via an oncogenic pathway characterised by defective DNA mismatch repair and MSI³⁰.

In comparison to microsatellite stable (MSS) tumours, those with MSI display a unique clinical and immunological phenotype. They are more likely to arise in the proximal colon, less commonly metastasise to lymph nodes and distant organs, and demonstrate better stage-specific survival^{182,244}. Importantly, their high mutational burden evokes a strong intra-tumoral lymphocytic reaction¹⁸⁰. The clinical significance of this intra-tumoral immunogenicity is the potential efficacy of immunotherapy with inhibitory checkpoint blockade.

An important advance in modern oncotherapeutics was the discovery of inhibitory checkpoints and their role in cancer immunology, recognised by the Nobel Prize in 2018. Inhibitory checkpoints are proteins expressed on the surface of activated T cells following T cell receptor (TCR) engagement with tumour antigens. Binding of inhibitory checkpoints and their ligands suppresses T cell effector function. Blockade of this interaction by targeted monoclonal antibodies preserves T cell effector function. Preclinical and clinical studies have demonstrated remarkable success, revolutionising anti-cancer therapy.

Programmed cell death protein-1 (PD-1) represents one of a number of inhibitory checkpoints. In CRC, clinical efficacy of PD-1 blockade has predominantly been limited to MSI tumours whilst MSS tumours are largely refractory^{137,216}. Following two landmark trials, KEYNOTE 016 and CheckMate 142, FDA approval was granted for anti-PD-1 agents pembrolizumab and nivolumab in select patients with CRC^{137,217,243}. T-cell immunoreceptor with Ig and ITIM domains (TIGIT), a less studied inhibitory checkpoint, but with promising potential, binds to its ligands CD155 and CD112 that are expressed by tumour cells and antigen-presenting cells in the tumour microenvironment. The TIGIT pathway has been shown to regulate T cell mediated tumour recognition and co-blockade of PD-1 enhances T cell anti-tumour function^{274,275}.

At present, immunotherapy is mainly considered in patients with metastatic MSI disease. It is notable however, that 1 in 5 MSS tumours are immunologically '*hot*' with high levels of T cell infiltration¹⁴⁵. On the basis of these data, it is hypothesised that a subset of MSS tumours may be immunologically similar to MSI tumours in terms of T cell infiltration and checkpoint expression. Hence a subset of patients with MSS tumours may respond to checkpoint therapy, and the spectrum of patients with CRC overall who may derive benefit may potentially be expanded. The aim of this study was investigate immune cell landscape and inhibitory checkpoint expression on tumour-infiltrating lymphocytes according to microsatellite status in patients with treatment naïve, non-metastatic colon cancer. A decision was made to

focus on colon cancer. The reasons for this decision were two-fold. Firstly, there is evidence to suggest that colon and rectal cancer represent distinct disease entities. The treatment paradigms for colonic and rectal tumours differ, and are largely based on the fact that they differ in terms of their recurrence patterns (which in turn suggests differences in tumour biology). Secondly, the majority of patients with locally advanced rectal cancer receive neoadjuvant chemoradiotherapy. Neoadjuvant chemoradiotherapy results in tumour regression and fibrosis, with complete regression achieved in approximately 20%. The effects of neoadjuvant therapy on the local immune response are unclear. Therefore, in an effort to keep the study population as homogeneous as possible, the focus of the study was patients with colon cancer. As early age onset CRC is relatively rare, patients of all ages were considered for inclusion in order to accrue sufficient data to allow a meaningful analysis.

7.2 Methods

7.2.1 Study population

A consecutive series of patients with histologically confirmed, non-metastatic, treatment naïve colorectal cancer undergoing surgery with curative intent in St. Vincent's University Hospital between 2018 and 2020 were prospectively studied. The study protocol was reviewed and approved by the institutional Research and Ethics Board. All samples were obtained with written informed consent.

Rectal cancer was defined as adenocarcinoma within 15cm from the anal verge on colonoscopy. Exclusion criteria included stage IV disease, neoadjuvant chemotherapy or radiotherapy, emergency presentation, or pre-existing immunosuppression secondary to autoimmune disorders or medication. Clinical staging was according to the 8th Edition of the American Joint Committee on Cancer tumour node metastasis (TNM) staging system and based on computed tomography (CT) of the thorax, abdomen and pelvis. Additionally, pelvic magnetic resonance imaging (MRI) was performed for local staging of rectal cancer. All patients were discussed at the institutional multi-disciplinary meeting. Baseline demographic, clinical, staging, and histopathological data were recorded.

7.2.2 Pathological analysis

The tumour stage was defined according to the TMN staging system and the American Joint Committee on Cancer classification.

Haematoxylin and eosin sections of the resected specimen were

analysed using a minimum dataset and a standardized reporting system. Microscopically clear resection (R0) was defined as a tumour-free resection margin of at least 1 mm. Microsatellite instability was assessed using immunohistochemistry for mismatch repair proteins, MLH1 (BD Bioscience, clone G168-728), PMS2 (BD Biosciences, clone A16-4), MSH2 (Calbiochem, clone FE11), and MSH6 (BD Biosciences, clone 44). Nuclear staining in any area of the tumour was classified as showing no loss of the mismatch repair proteins (MSS). Complete loss of nuclear staining of the mismatch repair proteins in the entire tumour along with positive staining of nuclei of non-neoplastic cells were classified as having loss of expression of that mismatch repair protein (MSI). Germline testing was performed as needed following patient counseling and consent.

7.2.3 Immunophenotyping

Immunophenotyping was performed by flow cytometry and single cell RNA sequencing. Sample collection, storage and processing was as outlined in Methods 2.7.3-4. Briefly, single-cell suspensions of tumour and healthy colonic tissue were obtained. A standardized digestion protocol of DNase 1 and collagenase IV was used. For intra-cytoplasmic cytokine staining, cells were stimulated *in vitro* with a standardized stimulation cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Dead cells were excluded by live/dead staining using Zombie Aqua (BioLegend). Cells were then stained on ice with antibodies against surface proteins (CD45, CD3, CD8, PD-1, TIGIT). TILs were defined as CD45⁺ CD3⁺ cells. Following extracellular

staining, cells were fixed and permeabilized using the eBioscience Transcription Factor Staining Buffer Set. Once fixation and permeabilisation was complete, intracellular staining was performed. All data were collected on a BD Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star). Single cell RNA sequencing was performed using the 10x genomics platform as described in Methods 2.6. Isolated subpopulations of T cells were evaluated.

7.2.5 Statistical analysis

Data are expressed as standard error of the mean (SEM). The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Categorical variables are reported numbers with percentages, and were assessed using χ^2 test or Fisher's exact test where appropriate. Differences were considered statistically significant at $p < 0.05$. Analyses were performed with Prism (GraphPad Prism version 7.0) and SPSS®version 26.0 (IBM, Armonk, New York, USA).

7.3 Results

7.3.1 Baseline demographics and clinical data

A total of 20 patients undergoing surgery with curative intent for treatment naïve, non-metastatic (clinical stage I-III) CRC were included. The median (range) age was 73 (41-87), and 60% were male. The majority of tumours (20, 95.2%) were located in the colon. One patient had synchronous colon (ascending colon) and rectal tumours. Three patients overall were aged under 50. Baseline demographics and clinical data are summarized in *Table 17*.

7.3.2 Microsatellite instability status

Of the 20 patients included, 6 (30.0%) demonstrated defined MSI. Lynch Syndrome was diagnosed in 2, whilst the remaining 4 had confirmed sporadic disease. The breakdown of pathological stage (pTNM) is outlined in *Table 17*.

Table 17. Baseline demographics and clinicopathological features

	N = 20
Median age (range)	73 (41-87)
Male sex	12 (60.0%)
Tumour site	
Caecum	3 (14.3%)
Ascending colon	2 (9.5%)
Transverse colon	5 (23.8%)
Hepatic flexure	1 (4.8%)
Sigmoid colon	9 (42.9%)
Rectum	1 (4.8%)
pTNM stage	
I	2 (9.5%)
II	9 (42.9%)
III	8 (38.1%)
MMR status	
MSI	6 (30.0%)
MSS	14 (70.0%)
Lynch Syndrome	2 (10%)

7.3.3 Inhibitory checkpoint expression

Expression of PD-1 and TIGIT on CD3+ cells was increased in tumour compared to healthy colon

Of the 20 patients included, 21 tumours were available for analysis, with one patient having 2 separate tumours (one colon tumour and one rectal tumour). Firstly, the expression of the inhibitory receptors PD-1 and TIGIT on CD3+ cells (T cells) in tumour and uninvolved healthy colonic tissue were analysed by flow cytometry. Expression of PD-1 and TIGIT were significantly increased in the tumour compared to uninvolved healthy colonic tissue among the entire study group

(Figure 16 & 17). Whilst CD3 expression of PD-1 and TIGIT was increased in tumour compared to healthy colon in all patients, the degree of expression varied markedly among the group, particularly for PD-1 (Figure 17). CD3+ PD-1+ cells could be divided into 3 distinct groups. Ranging from highest detected PD-1 expression to the lower limit of PD-1+, the gated PD-1+ CD3+ cells were divided into high PD-1 (PD-1^{high}), intermediate (PD-1^{int}) and low (PD-1^{low}) expression levels (Figure 18). Interestingly, the patient with synchronous tumours (both MSS), had low levels of PD-1 expression in the rectal tumour and intermediate levels of PD-1 expression in the colonic tumour. The TME contains distinct subpopulations of TILs with a range of PD-1 expression levels. Previous studies have demonstrated CD3+ PD-1^{high} expression is associated with exhaustion and hence poorer effector function. Categorizing PD-1 expression has several clinical implications. It may correlate with aggressiveness of the disease and clinical outcome, thus may provide prognostic information. Furthermore, PD-1 levels may represent a biomarker to predict anti-PD-1 therapeutic response.

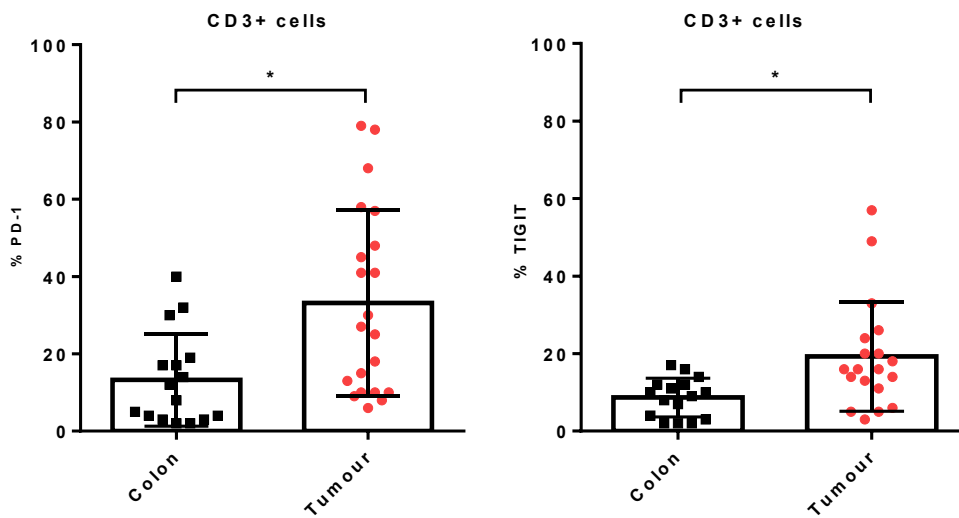


Figure 16. Percentage of CD3+ cells expressing PD-1 and TIGIT in uninvolved healthy colonic tissue and tumour. The percentage of CD3+ cells expressing PD-1 and TIGIT in healthy colon and tumour was evaluated in each patient by flow cytometry. Data are expressed as standard error of the mean (SEM). The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. * = $p < 0.05$

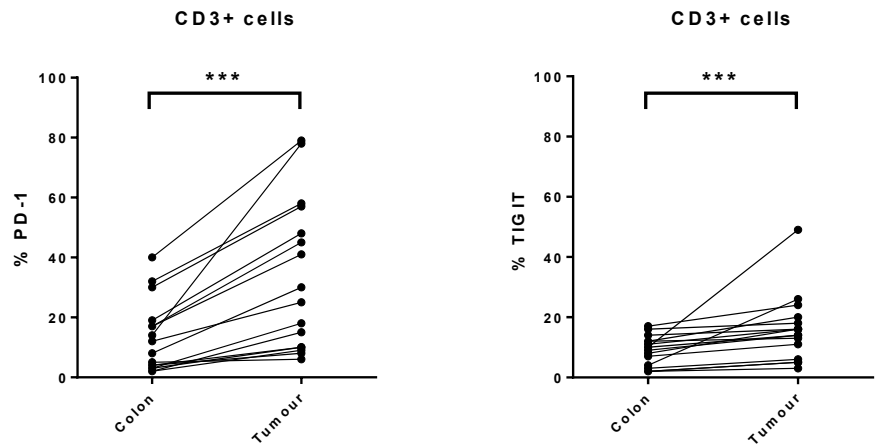


Figure 17. Percentage of CD3+ cells expressing PD-1 and TIGIT in paired uninvolved healthy colonic tissue and tumour samples.

The percentage of CD3+ cells expressing PD-1 and TIGIT in paired colon and tumour samples was evaluated by flow cytometry. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. *** = $p < 0.001$

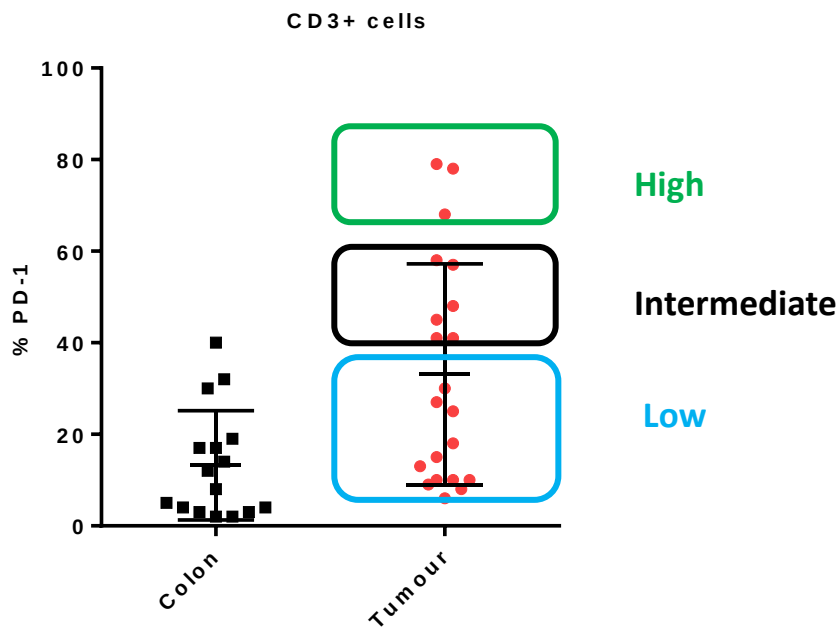


Figure 18. Percentage of CD3+ cells expressing PD-1 in uninvolved healthy colonic tissue and tumour. Ranging from highest detected PD-1 expression to the lower limit, the gated PD-1+ CD3+ cells were divided into low, intermediate and high levels of expression.

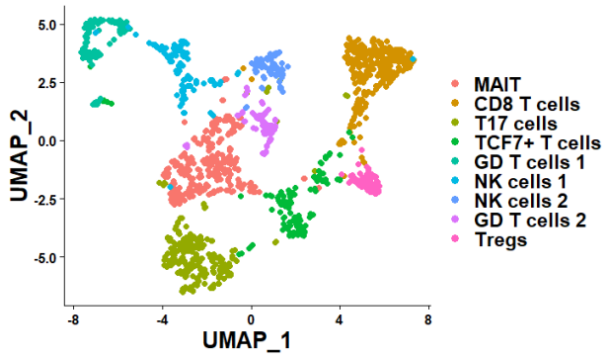
TILs in MSS tumours express inhibitory checkpoints

On the basis of the flow cytometry results, we sought to further define the immune landscape of colon cancer with single cell RNA sequencing. MSS tumours account for the majority of CRC and represent the greatest clinical challenge. Their immune profile remains poorly understood. Therefore, single cell RNA sequencing was only performed on MSS tumour samples (5 patients). The purpose of performing single cell analysis was firstly to evaluate the intra-tumoral lymphocyte response in MSS colon cancer, specifically

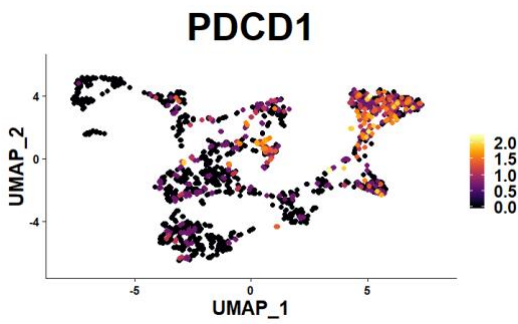
infiltration of various innate (natural killer (NK) cells, mucosal associated invariant T (MAIT) cells and $\gamma\delta$ T cells) and adaptive CD8⁺ and Tregs (CD4⁺) T cell subsets. Secondly, we sought to evaluate the expression of inhibitory checkpoints PD-1 and TIGIT on these CD3⁺ subsets. Thirdly, we evaluated CD3⁺ cell expression of several other inhibitory checkpoints including CTLA-4, Lymphocyte Activation Gene 3 (LAG3), and T cell immunoglobulin and mucin-3 (TIM-3 or HAVCR2). Evaluation of LAG3 and TIM-3 was on the basis of several preclinical and clinical tumour models demonstrating co-expression of LAG3 and TIM3 with PD-1, and synergistic immunosuppression²¹⁴. Co-expression of both checkpoint molecules reflected a more exhausted phenotype, with combined blockade more effective in controlling tumour growth than blocking either checkpoint alone.

On single cell analysis, CD8⁺ T cells, Tregs (CD4⁺), NK cells, MAIT cells and $\gamma\delta$ T cells were identified in the tumour samples (*Figure 17a*). These data demonstrate that MSS tumours ignite a robust intra-tumoral immune response and are not immunologically '*cold*'. The inhibitory checkpoints PD-1, TIGIT, CTLA4, LAG3 and TIM3 were expressed on both innate and adaptive T cells in the tumour but predominantly on CD8⁺ T cells and Tregs (*Figure 19 & 20*). Expression of inhibitory checkpoints implies T cell recognition of the tumour and activation, and further supports the flow cytometry findings that MSS tumours induce robust T cell responses (despite their lower mutational burden compared to MSI tumours).

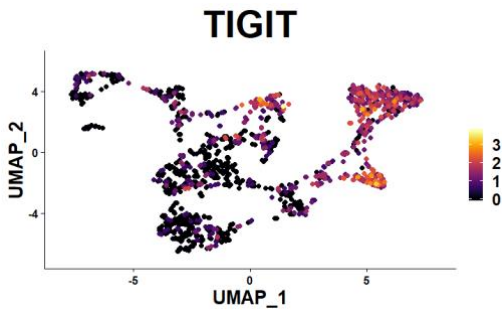
A



B



C



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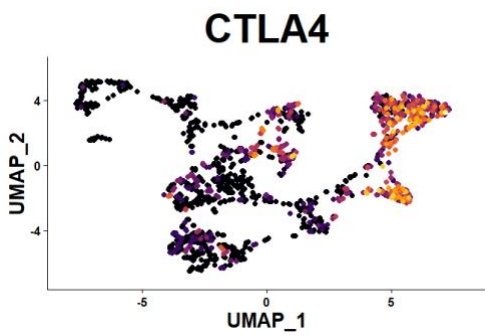
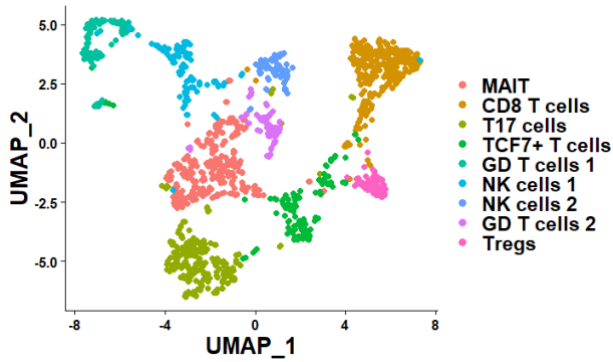


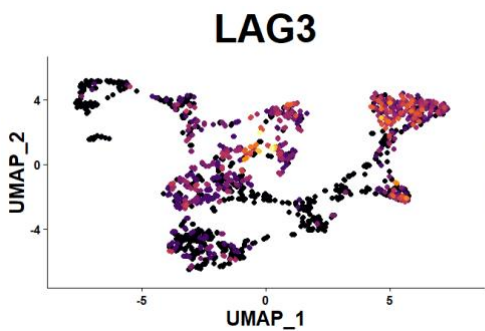
Figure 19. Single cell RNA sequencing showing subsets of tumour-infiltrating lymphocytes and inhibitory checkpoint expression.

Single cell RNA sequencing, using the 10x genomics platform, was performed on samples from 5 MSS tumours. Inhibitory checkpoint expression on isolated populations of T cells in the tumour samples was evaluated. The subsets of TILs identified in the tumour samples are illustrated in A. The inhibitory checkpoints PD-1, TIGIT, and CTLA4 expressed by the various subsets are illustrated in B-D respectively. The level of expression is indicated by the colour scale with the darker colour (black) indicating low expression and the brighter colour (yellow) indicating higher expression.

A



B



C

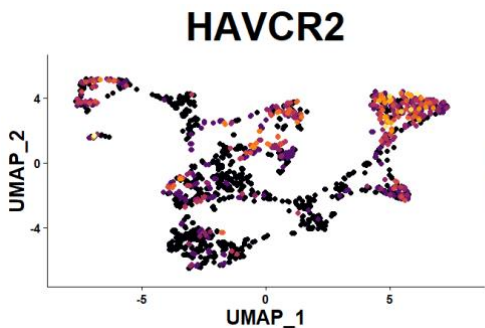
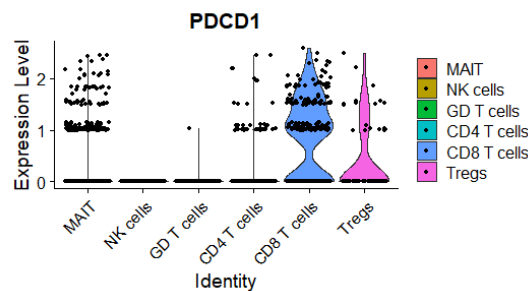


Figure 20. Single cell RNA sequencing showing TIL subsets and inhibitory checkpoint expression. The subsets of TILs identified in the tumour samples are illustrated in A. The inhibitory checkpoints LAG3 and TIM3 expressed by the various subsets are illustrated in B and C respectively. The level of expression is indicated by the colour scale with the darker colour (black) indicating low expression and the brighter colour (yellow) indicating higher expression.

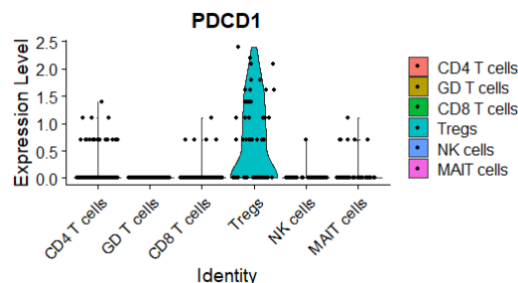
Inhibitory checkpoints were predominantly expressed on Tregs in healthy colon

Inhibitory checkpoint expression on CD3⁺ cells in healthy colon was also evaluated by scRNA sequencing. PD-1 and CTLA4 were predominantly expressed on Tregs which is expected given the important role of Tregs in maintaining homeostasis and preventing autoimmunity in the gastrointestinal tract. TIM3 was preferentially expressed by NK cells and $\gamma\delta$ T cells, whilst several CD3⁺ subsets expressed TIGIT and LAG3. It is possible the TIGIT pathway in particular may play an important role in maintaining tissue homeostasis in healthy colon. (Figure 21-23)

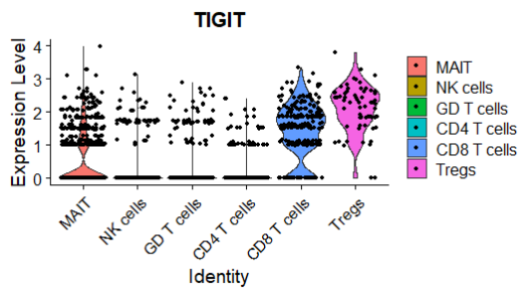
A. Tumour



B. Colon



C. Tumour



D. Colon

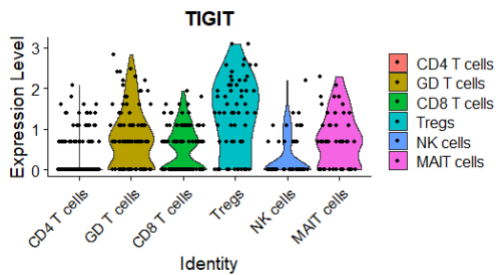
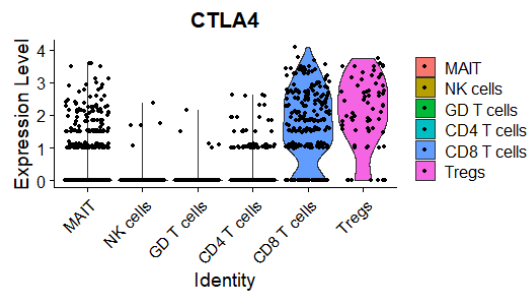


Figure 21. PD-1 and TIGIT expression on CD3+ subsets in tumour and healthy colon. Single cell RNA sequencing, using the 10x genomics platform, was performed on colon and tumour samples from 5 patients with MSS tumours. PD-1 and TIGIT expression on isolated populations of T cells in the tumour samples and colon were evaluated.

A. Tumour



B. Colon

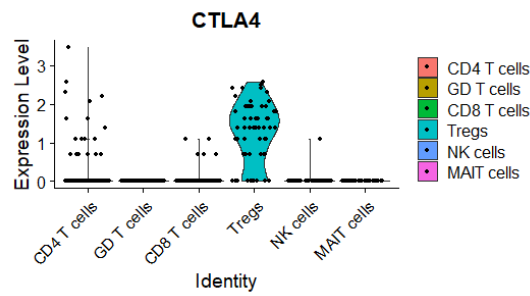
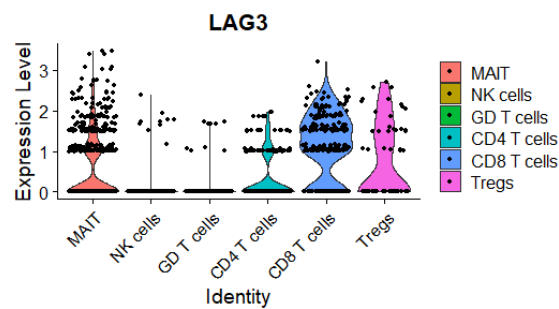
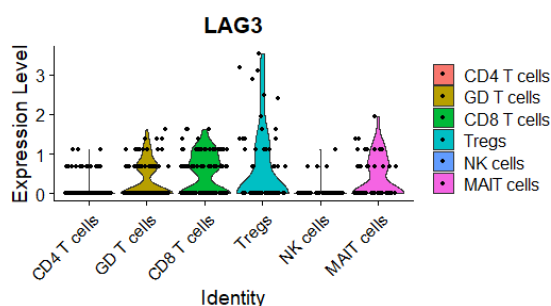


Figure 22. CTLA4 expression on CD3+ subsets in tumour and healthy colon. Single cell RNA sequencing, using the 10x genomics platform, was performed on colon and MSS tumour samples. CTLA4 expression on isolated populations of T cells in the tumour samples and colon were evaluated.

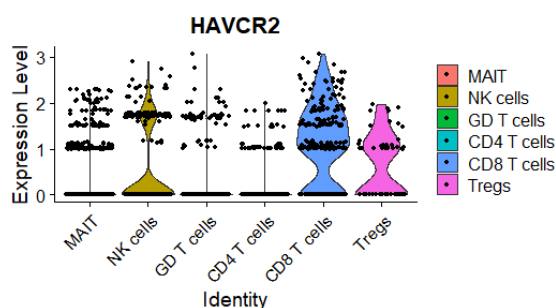
A. Tumour



B. Colon



C. Tumour



D. Colon

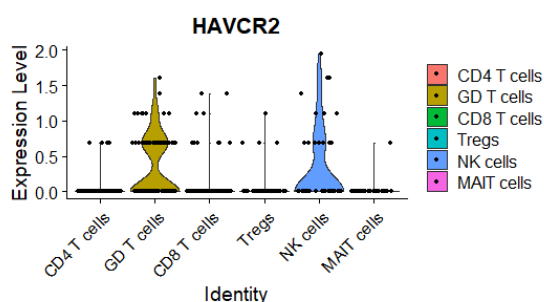


Figure 23. LAG3 and TIM3 expression on CD3+ subsets in tumour and healthy colon. Single cell RNA sequencing, using the 10x genomics platform, was performed on healthy colon and MSS tumours. LAG3 and TIM3 expression on isolated populations of T cells in the tumour and colon were evaluated.

T cell inhibitory checkpoint expression according to MSI status

Analysis of inhibitory checkpoint expression on the basis of MSI status revealed significantly increased expression of PD-1 in MSI

tumours overall compared to MSS (*Figure 24*). In view of the increased clinical efficacy of PD-1 blockade in patients with MSI colon cancer, this finding was expected. In the uninvolved healthy colonic tissue, higher expression of PD-1 was also observed in the MSI group compared to the MSS group (*Figure 24*). This finding may reflect a field change at organ or at least segmental level, whereby changes in the immune milieu extend beyond the immediate tumour microenvironment.

The degree of upregulation of PD-1 in the tumour however, varied markedly among patients with the same MSI status. The variation in the MSI group was particularly interesting given that MSI tumours are generally considered to be immunologically ‘hot’. These data suggest that in fact not all MSI tumours are immunologically homogenous which may have implications when selecting patients suitable for checkpoint blockade. It appears there is a degree of overlap with a subset of MSI tumours that are ‘MSS like’, and a subset of MSS tumours that are ‘MSI like’ (*Figure 25*).

There was no significant difference in expression of TIGIT of CD3+ cells in tumour or healthy colon among both groups (*Figure 26*). It is possible that the PD-1/PD-L1/2 axis plays a greater role in colon cancer for reasons not yet known. Analysis of expression of PD-1 and TIGIT in paired tumour and colon samples found increased expression in tumour compared to colonic tissue (*Figure 27*).

At present, no molecular criteria other than MSI are required to be considered for anti-PD-1 therapy. Clinical studies have demonstrated varying responses to therapy among patients with MSI, however these variations have not yet been explained on a molecular level. Similarly, heterogeneity in PD-1 expression exists among the MSS tumours. A proportion (approximately 1 in 4) exhibit levels of PD-1 expression comparable to the MSI group. Furthermore, CD3+ PD-1+ cells in the tumour samples could be divided into 3 distinct subsets in both the MSI and MSS groups: high PD-1^{high}, intermediate (PD-1^{int}) and low (PD-1^{low}) expression levels. (*Figure 28*). These findings support the hypothesis that a subset of MSS tumours may be immunologically similar to MSI tumours in terms of T cell infiltration and checkpoint expression, and hence may respond to checkpoint therapy.

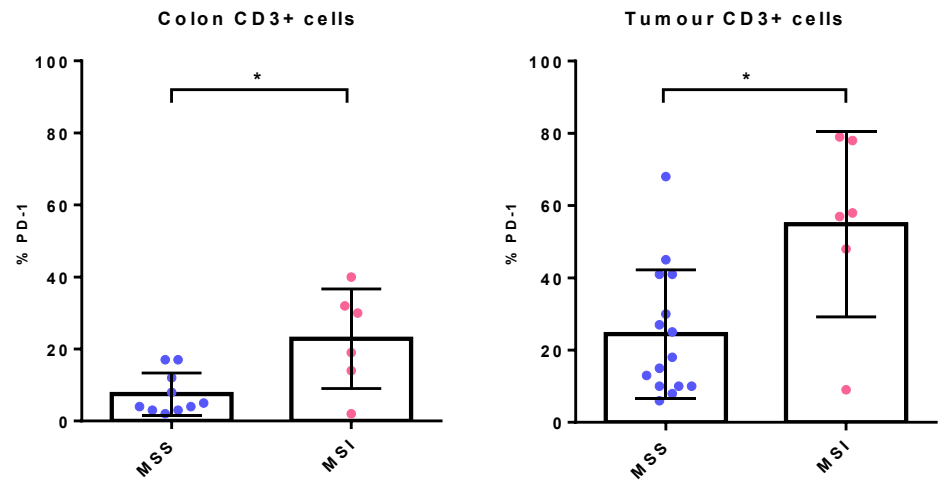
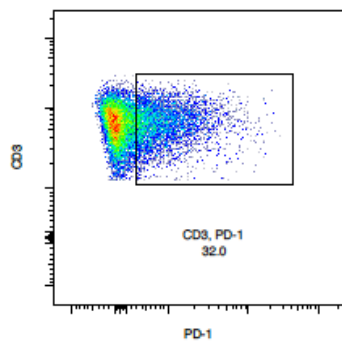
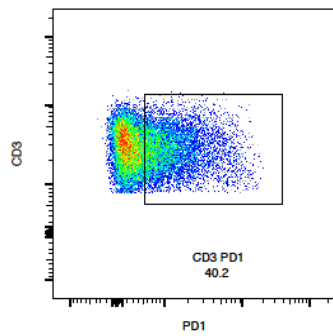


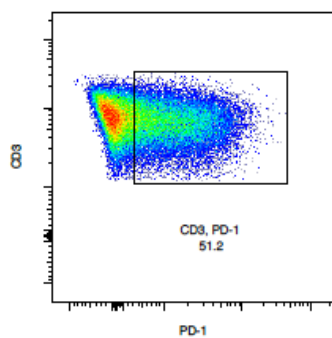
Figure 24. Percentage of CD3+ cells expressing PD-1 in uninvolved healthy colonic tissue and tumour according to MSI status. The percentage of CD3+ cells expressing PD-1 in healthy colon and tumour was evaluated in each patient by flow cytometry. Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. * = $p < 0.05$



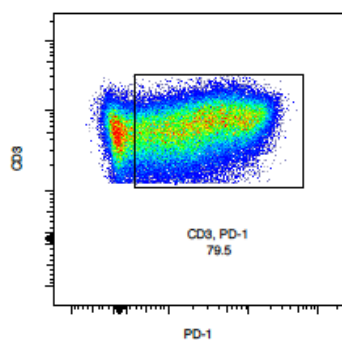
A.



B.



C.



D.

Figure 25. Representative flow plot of CD3+ cells in tumour

expressing PD-1. A. This plot represents what is considered a typical MSS tumour with low PD-1 expression. B. This plot represents an ‘MSS like’ MSI tumour with intermediate PD-1 expression. C. This plot represents an ‘MSI like’ MSS tumour with intermediate PD-1

expression. D. This plot represents a typical MSI tumour with high PD-1 expression.

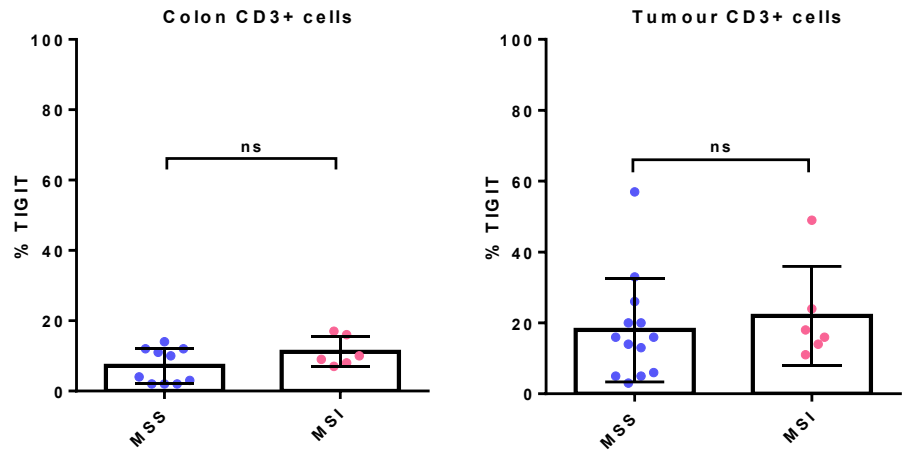


Figure 26. Percentage of CD3+ cells expressing TIGIT in uninvolved healthy colonic tissue and tumour according to MSI status. The percentage of CD3+ cells expressing TIGIT in healthy colon and tumour was evaluated in each patient by flow cytometry. Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. NS = $p > 0.05$

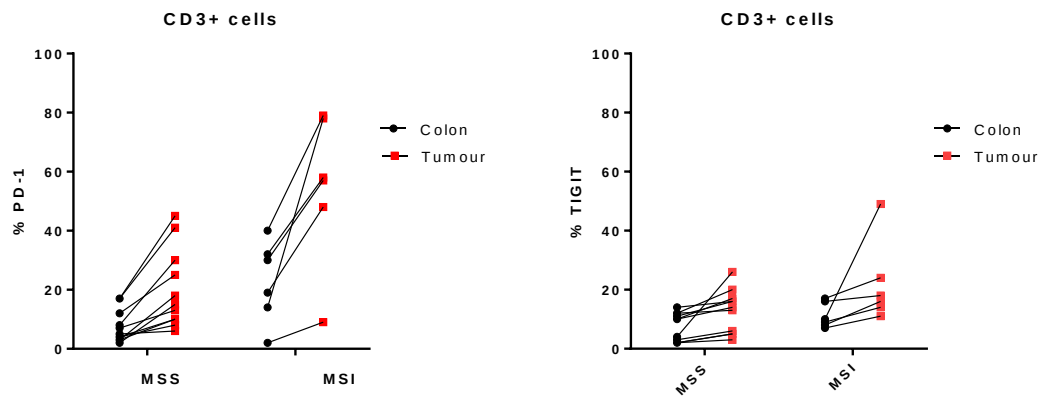


Figure 27. Percentage of CD3+ cells expressing PD-1 and TIGIT

in paired uninvolved healthy colonic tissue and tumour. The

percentage of CD3+ cells expressing PD-1 and TIGIT in paired colon and tumour samples was evaluated by flow cytometry. The dot plots represent each individual patient sample.

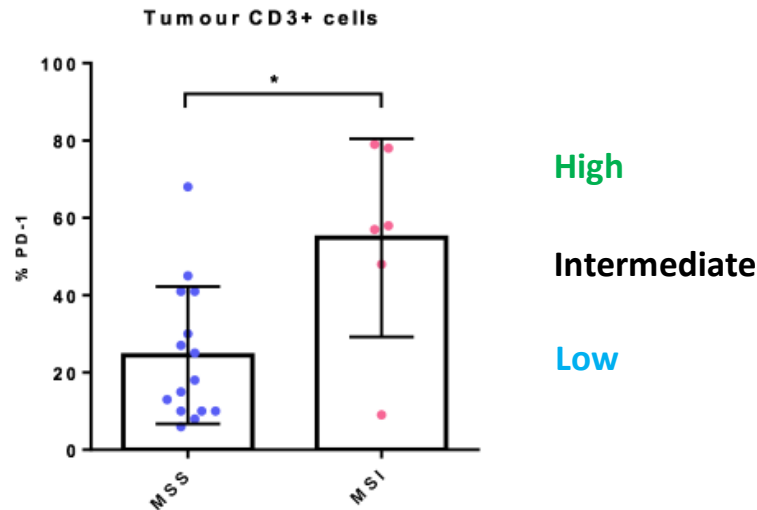


Figure 28. Patients with MSI tumours have increased PD-1 expression on intra-tumoral T cells. The percentage of CD3+ cells expressing PD-1 in tumour was evaluated by flow cytometry. Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Ranging from highest detected PD-1 expression to the lower limit, the gated PD-1+ CD3+ cells were divided into low, intermediate and high levels of expression for MSI and MSS tumours.

7.3.4 Functional analysis of TILs

Following assessment of inhibitory checkpoint expression on TILs, we investigated the functional properties of these TILs. To define the functional properties, T cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C and assessed for the ability to produce IFN γ , IL-17a and amphiregulin (AREG). IFN γ represents a well-defined marker of cytotoxicity, whilst IL-17a and AREG play critical roles in mucosal tissue repair with recent studies suggesting a link between wound healing and cancer

development. As CRC is associated with a wound-healing immune phenotype, we sought to evaluate T cell production of these mediators¹⁷⁶.

Tumoral CD3+ PD-1+ cells produced less IFN γ than CD3+ PD-1- cells

IFN γ was evaluated as a marker of cytotoxicity in tumour and uninvolved healthy colonic tissue. Firstly, the percentage of CD3+ cells producing IFN γ in uninvolved healthy colon and tumour did not differ (*Figure 29*). As PD-1 expression may either indicate tumour specific T cell activation, or a dysfunctional state of ‘exhaustion’, co-expression of PD-1 and IFN γ was analysed. Both PD1+ and PD1- CD3+ cells in the tumour produced IFN γ . T cells that expressed PD-1 produced more IFN γ than PD-1- T cells suggesting that PD-1 expression represented a marker of activation rather than exhaustion on tumoral T cells (*Figure 30*). This finding highlights the ambiguous role of PD-1 in defining effective or ineffective T cell responses. Identification of activated or exhausted T cells is not possible on the basis of inhibitory checkpoint expression alone. Both TIGIT+ and TIGIT- T cells also produced IFN γ but T cells that expressed TIGIT produced less IFN γ suggesting TIGIT expression on tumoral T cells represents a marker of exhaustion. T cell production of IFN γ was also compared according to MSI status. No significant differences in the tumour were observed between groups but CD3+ IFN γ production was higher in healthy colon in the MSS group (*Figure 31*). These

findings suggest that MSI status does not appear to influence T cell functionality.

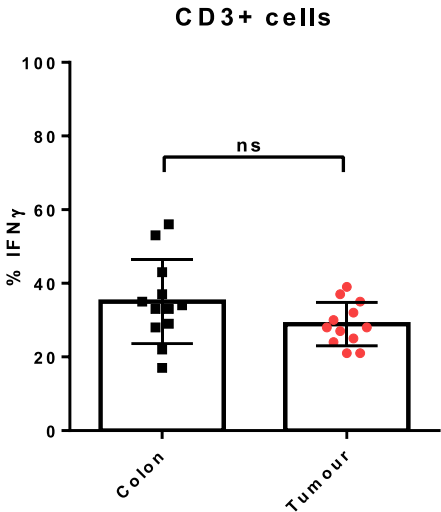


Figure 29. Percentage of CD3+ cells in uninvolved healthy colon and tumour producing IFN γ . The percentage of CD3+ cells producing IFN γ in healthy colon and tumour was evaluated in each patient by flow cytometry. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C and assessed for the ability to produce IFN γ . Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. NS = $p > 0.05$

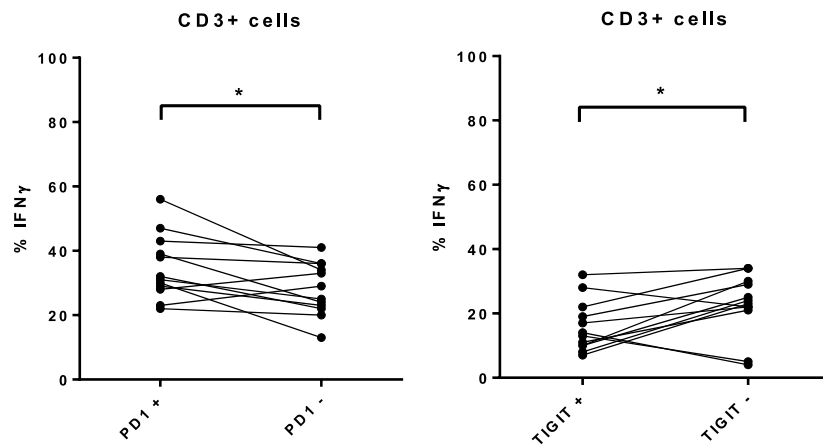


Figure 30. Tumoral CD3+ cell production of IFN γ on the basis of PD-1 and TIGIT expression. The percentage of CD3+ PD1+ and CD3+ PD1- cells in tumour producing IFN γ was evaluated by flow cytometry. The percentage of CD3+ TIGIT+ and CD3+ TIGIT- cells in tumour producing IFN γ was also evaluated. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C to assess ability to produce IFN γ . Wilcoxon matched pairs signed rank test was used for statistical analysis. * = p < 0.05

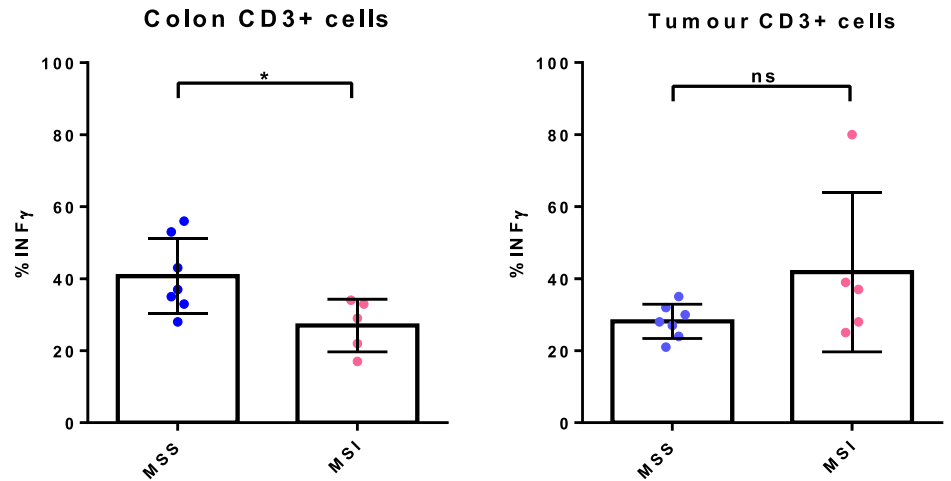


Figure 31. Percentage of CD3+ cells in uninvolved healthy colon and tumour producing IFN γ according to MSI status. The

percentage of CD3+ cells in healthy colon and tumour producing IFN γ was evaluated by flow cytometry. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C to assess ability to produce IFN γ . Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. * = $p < 0.05$. NS = $p > 0.05$

Measurement of CD3+ production of IL-17a and AREG in tumour and healthy colon did not differ according to MSI status

Due to their role in wound healing and cancer development, as well as their specific relevance in CRC, IL-17a and AREG production were evaluated in tumour and uninvolved healthy colonic tissue. Production of IL-17a and AREG in tumour and colon did not differ (*Figure 32*). The percentage of CD3+ cells producing IL-17a and AREG in tumour

and colon were also compared according to MSI status. No significant differences were observed between groups for IL-17a (*Figure 33*). For AREG, increased production in colonic tissue was observed in the MSS group compared to the MSI group (*Figure 34*). AREG production in the tumour did not differ between the two groups (*Figure 34*). In conclusion, CD3+ production of IL-17a and AREG did not differ between patients with MSI or MSS tumours.

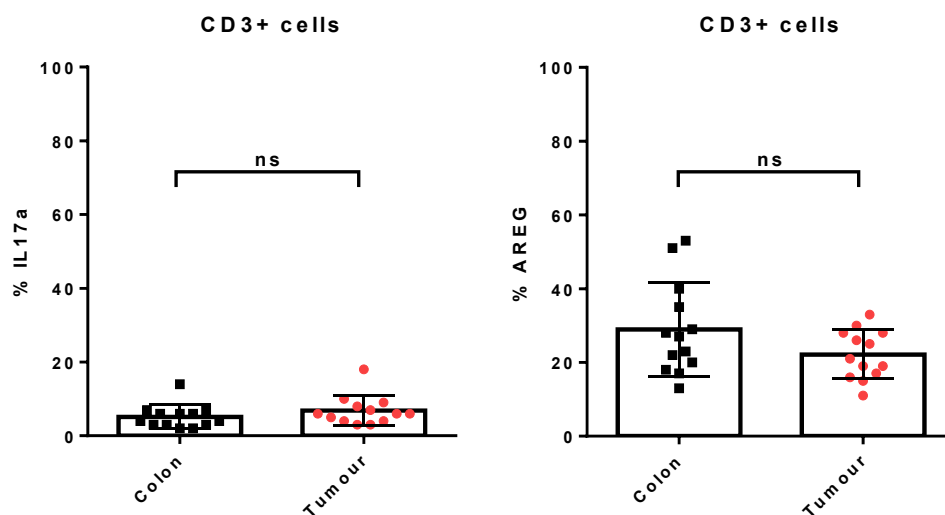


Figure 32. Percentage of CD3+ cells in the tumour producing IL-17a and amphiregulin. The percentage of CD3+ cells producing IL17a and AREG in healthy colon and tumour was evaluated by flow cytometry. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. NS = $p > 0.05$

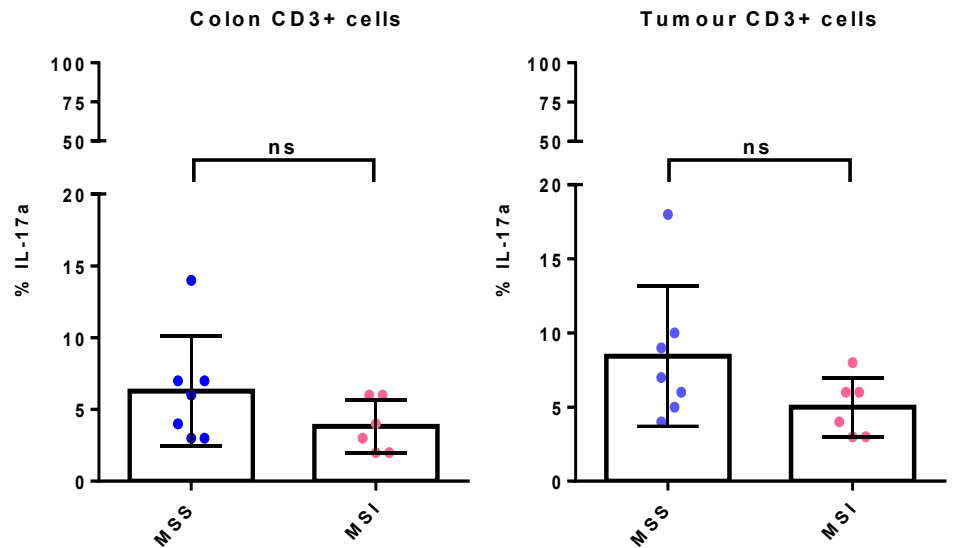


Figure 33. Percentage of CD3+ cells in the tumour producing IL-17a according to MSI status. The percentage of CD3+ cells producing IL17a in healthy colon and tumour was evaluated by flow cytometry. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C and assessed for the ability to produce IL17a. Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. NS = $p > 0.05$

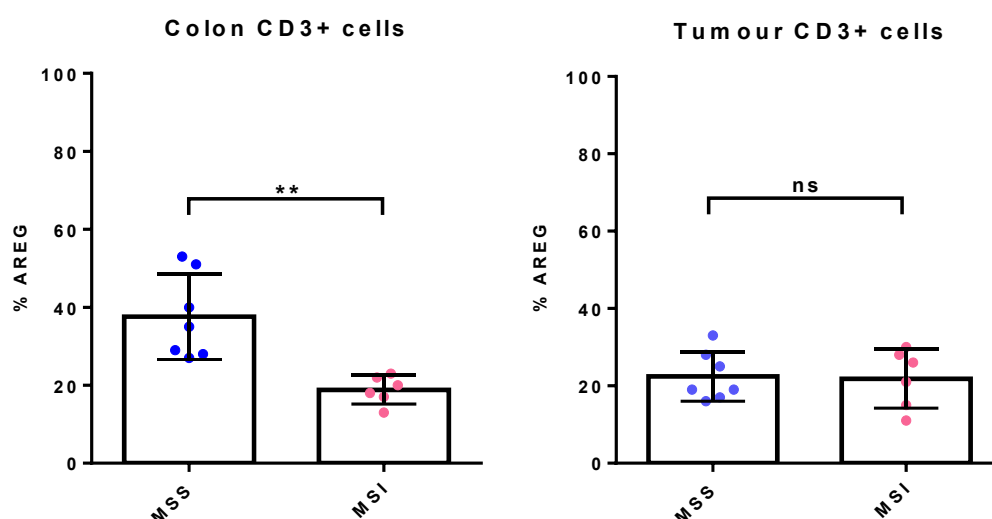
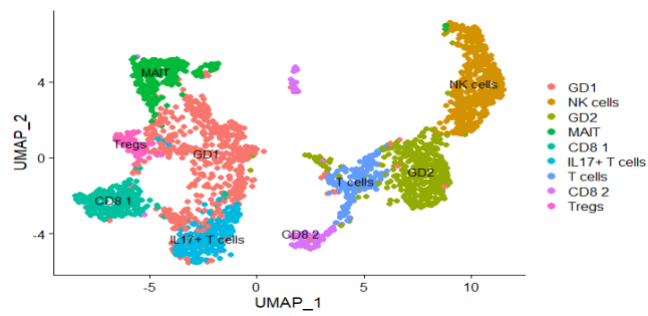


Figure 34. Percentage of CD3+ cells in the tumour producing

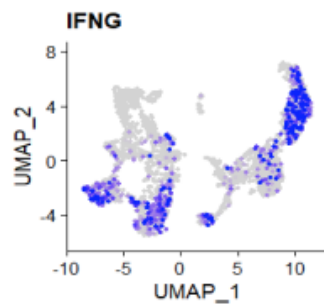
AREG according to MSI status. The percentage of CD3+ cells producing AREG in healthy colon and tumour was evaluated by flow cytometry. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Patients with MSI tumours were compared to those with MSS tumours. Data are expressed as SEM. The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. ** = $p < 0.01$. NS = $p > 0.05$

Single cell RNA sequencing

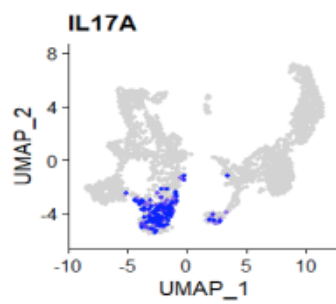
In order to define which T cell subsets were producing IFN γ , IL-17a and AREG, single cell RNA sequencing was performed on five MSS colon tumours. This showed that several subsets of TILs produced varying levels of IFN γ , IL-17a and AREG. IFN γ was mainly produced by CD8+ T cells and NK cells, whilst AREG was predominantly produced by $\gamma\delta$ T cells (Figure 35 & 36).



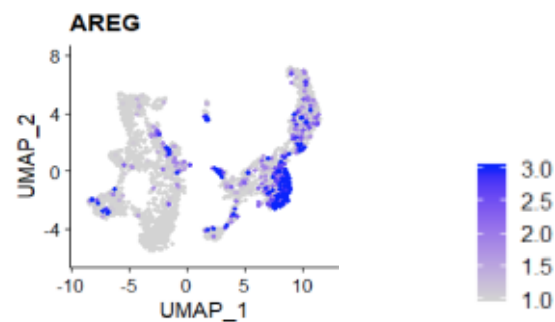
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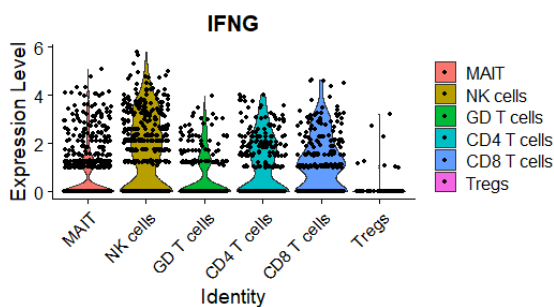
C.



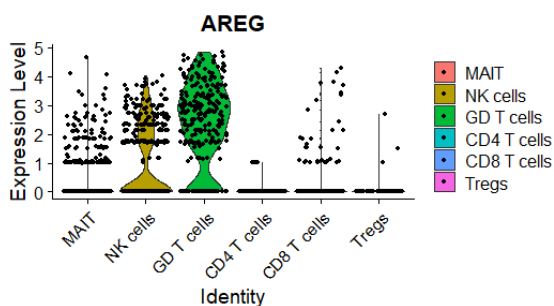
D.

Figure 35. Single cell RNA sequencing of colon tumours showing subsets of TILs. Single cell RNA sequencing, using the 10x genomics platform, was performed on samples from 5 MSS tumours.

Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Production of IFN γ , IL-17a and AREG by isolated populations of T cells in the tumour samples was evaluated (A). Production by the various subsets is illustrated in B-D. The level of expression is indicated by the colour scale with the lighter colour (grey) indicating low expression and the brighter colour (purple) indicating higher expression.



A.



B.

Figure 36. Single cell RNA sequencing of colon tumours showing

subsets of TILs. Single cell RNA sequencing, using the 10x genomics platform, was performed on samples from 5 MSS tumours. Cells were stimulated with a standardized cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Production of IFN γ ,

(A) and AREG (B) by isolated populations of T cells in the tumour samples was evaluated.

7.3.5 Association between PD-1 expression and clinicopathological parameters

The clinicopathological features of patients with high or intermediate PD-1 expression on flow cytometry were compared to those with low PD-1 expression. PD-1 was significantly associated with MSI but not with age, sex, tumour location, pathological stage or histopathological features such as lymphovascular invasion, extramural venous invasion, perineural invasion, differentiation or tumour budding. Clinicopathological features are summarized in *Table 18*.

Table 18. Comparison of clinicopathological features according to PD-1 expression

	PD-1 High/Intermediate N = 9	PD-1 Low N = 12	p
Median age (range)	61 (40-81)	73 (51-89)	0.122
Male, N (%)	5 (55.5%)	7 (58.3%)	1.000
Tumour location Proximal colon Distal colon/rectum	7 (77.8%) 2 (22.2%)	4 (33.3%) 8 (66.7%)	0.080
pTNM stage I II III	0 (0%) 5 (62.5%) 3 (37.5%)	2 (16.6%) 5 (41.7%) 5 (41.7%)	0.486 0.387 1.000
Differentiation Poor Moderate Well	3 (33.3%) 6 (66.7%) 0 (0%)	2 (16.7%) 10 (83.3%) 0 (0%)	0.611
LVI	3 (33.3%)	4 (33.3%)	1.000
EMVI	2 (22.2%)	4 (33.3%)	0.659
Perineural invasion	0 (0%)	2 (16.7%)	0.486
Mucin 0-10% 10-50% >50%	7 (77.8%) 1 (11.1%) 1 (11.1%)	12 (100%) 0 (0%) 0 (0%)	0.171
Tumour Budding	3 (33.3%)	4 (33.3%)	1.000
MSI	5 (55.5%)	1 (8.3%)	0.046

7.4 Discussion

The development of checkpoint inhibitors to block immune checkpoint receptors and their ligands has achieved remarkable success in several solid organ malignancies, including CRC. Clinical efficacy however, varies significantly among patients. A greater understanding of the complex interplay between the tumour and immune microenvironment is needed to guide patient selection. The present study evaluated checkpoint expression on TILs in treatment naïve CRC according to MSI status.

Firstly, significantly increased expression of PD-1 and TIGIT was observed in the tumour compared to uninvolved healthy colonic tissue among the entire study group. Upregulation of inhibitory checkpoints indicates T cell recognition of tumour. Yet due to various mechanisms enabling evasion of immunosurveillance, the tumours are not eradicated. In keeping with previous reports, expression of PD-1 was significantly upregulated in MSI tumors relative to MSS. The higher levels of PD-1 expression in MSI tumours is in line with the markedly better response to PD-1 blockade observed in these tumours in clinical trials. However, a subset of MSS tumours (1 in 4) demonstrated levels of PD-1 expression comparable to MSI tumours. Interestingly, TIGIT expression did not differ significantly between groups.

The potential clinical implications of these findings are several-fold. Firstly, a subset of MSS tumours exist may respond to immunotherapy with checkpoint blockade. At present, immunotherapy is not routinely

considered in patients with MSS disease owing to the poor response observed in clinical trials. These tumours represent a significant clinical challenge as their immune microenvironment remains poorly understood. Limited response to checkpoint therapy has led to the assumption that they are immunologically '*cold*' with low inhibitory checkpoint expression. Their low mutational burden is thought to contribute to a weaker local immune response compared to MSI tumours. The data in this study suggest a subset of '*hot*' or 'MSI-like' MSS tumours exist, and that the spectrum of patients who may benefit from checkpoint therapy may potentially be expanded.

Furthermore, MSI tumours are not immunologically homogenous in terms of inhibitory checkpoint expression. Marked variations in PD-1 expression in particular were observed among the MSI group. Where that is the case, clinical response to checkpoint blockade will also vary. A recent single centre study evaluating neoadjuvant PD-1 blockade in MSI CRC reported a pCR rate of 65% with toripalimab (anti-PD-1 monoclonal antibody), and a pCR rate of 88% in those treated with toripalimab plus celecoxib²⁷⁶. In the KEYNOTE-177 trial, no significant difference in overall survival was observed between patients who received pembrolizumab or chemotherapy for treatment naïve metastatic CRC^{277,278}. As such dichotomization of CRC on the basis of MSI status is limited. MSI instead represents an umbrella term relating to a group of tumours that demonstrate clinical, histopathological, and immunological heterogeneity. Thus MSI status in isolation may not represent the most accurate method of assessing

suitability for immunotherapy or predicting response. Molecular selection criteria for checkpoint therapy will need to expand beyond this binary classification system.

CRC is a complex disease composed of several heterogeneous subtypes, each characterised by different genetic and epigenetic alterations. Using gene expression analysis, it has more recently been further subdivided into four consensus molecular subtypes on the basis of distinguishing clinical behaviour, and underpinning biological, molecular and immune characteristics to guide prognosis and management³⁰. Expression of PD-1 alone cannot be used to distinguish T cell responses as it may represent a marker of T cell activation or dysfunction. Although a TME characterised by the accumulation of PD-1+ CD8+ activated effector T cells, the presence of IFN- γ , and the expression of PD-L1 by tumour cells is associated with response to anti-PD-1 therapy, identification of genetic and epigenetic changes in TCR signaling is required to distinguish these two functional states and predict response to PD-1 blockade^{279,280}.

Intra-tumoral immune response is influenced by tumour biology. In particular, tumour mutational burden is an important predictor of tumour immunogenicity. MSI tumours have a higher mutational rate than MSS. These somatic mutations lead to the formation of neoantigens which promote T-cell activation and infiltration¹⁷⁷. Importantly, a subset of MSS tumours (3%) harbor DNA polymerase epsilon or delta (POLE, POLD) mutations. These POLE-mutated

MSS tumors have an even higher neoantigen load than MSI tumours, and exhibit strong immune infiltration²⁸¹⁻²⁸³. Clinical response to pembrolizumab (anti-PD-1 mAb) has been observed in POLE-mutated MSS CRC, thus POLE mutations may be predictive markers of response to checkpoint blockade in CRC²⁸⁴. The ongoing phase II ARETHUSA trial is evaluating whether a priming course of temozolomide chemotherapy may sensitise patients with MSS mCRC to anti-PD-1 inhibitor pembrolizumab. Temzolomide has been shown to inactivate MMR genes, leading to an increase in TMB and immunogenic neoantigens.

In addition to mutational load, tumor heterogeneity has also been proposed as a parameter to stratify patients for targeted immunotherapy. Tumour heterogeneity refers to the presence of distinct populations of cells within a tumour that display different molecular and phenotypical profiles. Hypermutated tumours (MSI and POLE-mutated MSS) display marked heterogeneity. The higher load of neoantigens in these tumors stimulates a profound antitumor immune response²⁸⁵. These tumours are characterized by depletion of immunosuppressive cells and upregulation of inhibitory checkpoints. Similar immune responses however, have been observed in a subset of homogeneous non-hypermutated tumours²⁸⁶. Theoretically, they may respond to checkpoint blockade. The mechanisms driving immune responses in these tumours are as yet undefined. Undoubtedly, additional unknown parameters besides mutational burden and

heterogeneity contribute to the profile of the immune microenvironment.

Finally, should intra-tumoral immune profile be incorporated into CRC staging to guide management? If so, how should the immune landscape be categorized? The Immunoscore has been proposed as a new component of a TNM-Immune classification having been validated as a strong predictor of clinical outcome (irrespective of MSI status)¹⁴⁵. Evidently, the immune microenvironment is far more complex than a binary '*hot*' and '*cold*' classification system based on density of T cell infiltration. Instead it is characterized by heterogeneous and complex immune signatures characterized by checkpoint expression, and pro- and anti-tumour mediators^{175,176}. In the present study, TIL production of IFN γ , IL-17a, and AREG was evaluated. Although both pro-tumorigenic and anti-tumorigenic properties have been observed, IFN γ is generally considered a key effector molecule of the anti-tumour immune response, whilst IL-17a is a pro-inflammatory cytokine which has an important role in tumorigenesis and metastases. AREG, the ligand of the epidermal growth factor receptor (EGFR), is expressed by a variety of TIL subsets (CD8⁺ T cells, CD4⁺ Tregs) and promotes immune suppression in the tumour microenvironment. No significant differences in IFN γ , IL-17a, and AREG expression were observed between the MSI and MSS groups, however expression did vary among patients with the same MSI status. The IFN γ , IL-17a and

AREG signatures of the immune microenvironment may have a role in immunotherapy sensitivity and the design of novel agents.

The challenge is to identify tumour and immune biomarkers predictive of response. The dichotomisation of CRC MSI and MSS is limited, and tumour biology needs to be further defined to stratify patients. Similarly, the immune microenvironment needs to be further profiled in order to accurately select patients exhibiting immune responses that can be successfully targeted. Although mutational burden is a biomarker of response to checkpoint blockade, not all MSI tumours respond. Similarly, while density of T cell infiltration is predictive of clinical outcome, it alone does not predict therapeutic efficacy. Even if MSS tumours are infiltrated by T cells, they will not respond to checkpoint therapy if TILs do not express inhibitory checkpoints. Even then, sole expression of PD-1 cannot be used to distinguish T cell responses as it may represent a marker of T cell activation or dysfunction. Although a TME characterised by the accumulation of PD-1+ CD8+ activated effector T cells, the presence of IFN- γ , and the expression of PD-L1 by tumour cells is associated with response to anti-PD-1 therapy, identification of genetic and epigenetic changes in TCR signaling is required to distinguish these two functional states and aid in predicting response to PD-1 blockade^{279,280}. In the present study, no clinical or histopathological features (other than MSI) were associated with PD-1 expression. Flow cytometric analysis and single cell RNA sequencing are not feasible on a large scale in clinical practice. Immunohistochemistry staining of specific markers (such as

PD-1) may represent a more realistic option that could be incorporated into routine histopathological analysis of diagnostic biopsies, and ultimately become part of a standardized reporting system.

The impact of the immune landscape of uninvolved colon in clinical outcome and response to immunotherapy is unclear. Interestingly, PD-1 expression in uninvolved healthy colon was higher in the MSI group, whilst AREG production was higher in the MSS group. The former suggests immune activation while the latter is associated with immunosuppression.

Differences in checkpoint expression may have significant implications in defining patient subgroups potentially responsive to checkpoint blockade. Understanding the tumour and immune microenvironment will enable more accurate selection of patients likely to derive a meaningful benefit, in addition to enhancing therapeutic effect. Historically, the focus of checkpoint blockade has been predominantly on MSI tumours. As 85% of CRCs are MSS, future emphasis should be placed on identifying those tumours in this group who may respond.

Chapter 8: Functionally distinct $\gamma\delta$ T cells in colorectal cancer

Abstract

8.1 Background

8.2 Methods

8.2.1 Study population

8.2.2 Pathological analysis

8.2.3 Immunophenotyping

8.2.4 Statistical analysis

8.3 Results

8.3.1 Enrichment of V δ 1 cells in tumour compared to healthy colon

8.3.2 Inhibitory checkpoint expression and cytotoxicity of intra-tumoral $\gamma\delta$ T cells

8.3.3 Functional heterogeneity of intra-tumoral $\gamma\delta$ T cells

8.3.4 $\gamma\delta$ T cell expression of AREG in healthy colon

8.3.5 AREG expression is associated with NKp80+ V δ 1 cells

8.4 Discussion

Abstract

Background: $\gamma\delta$ T cells are an important tissue resident population of innate T cells in the gastrointestinal tract. Although associated with a good prognosis in most solid organ tumours, they do not appear to represent a favourable prognostic indicator in colorectal cancer. The aim of this study was to profile $\gamma\delta$ T cells in colon tumours and adjacent healthy colon.

Methods: Single-cell RNA sequencing, using the 10x genomic platform, and flow cytometric analysis were performed *ex vivo* on tumour and uninvolved colonic tissue samples from patients undergoing surgical resection for treatment naïve colon cancer. The functional status of isolated tissue-resident $\gamma\delta$ T cells was analysed.

Results: A subset of $\gamma\delta$ T cells (V δ 1) were found to be enriched in tumour compared to healthy colonic tissue. Functionally distinct subpopulations of V δ 1 cells in the tumour were identified. One subset produced IFN γ , demonstrating a cytotoxic phenotype. The other subset demonstrated a wound healing phenotype, producing amphiregulin (AREG). NKp80 (*KLRF1*) was strongly associated with AREG expression, while TIGIT was associated with the cytotoxic phenotype.

Conclusion: A subset of $\gamma\delta$ T cells produce AREG in human colon tumours, which can promote tumour growth and contribute to an immunosuppressed tumour microenvironment. Enhancing the proportion of cytotoxic V δ 1 $\gamma\delta$ T cells and depleting the proportion of wound healing V δ 1 $\gamma\delta$ T cells represents a novel immunotherapeutic strategy in colorectal cancer.

8.1 Background

The immune landscape of CRC has important predictive and prognostic implications on clinical outcome^{143,181}. Intra-tumoral lymphocyte density has been internationally validated as an independent predictor of disease-specific survival¹⁴⁵. Various immune signatures correlating with prognosis have also been identified^{175,176}. The tumour microenvironment consists of many types of immune cell populations, each with varying functions and impact on disease-related outcome. Analysis of specific cell types in 18,000 tumours across 39 cancer types found that $\gamma\delta$ T cells were the most prognostically favourable immune cells²⁸⁷. Unlike many other tumour-infiltrating lymphocytes (TILs) such as CD8+ and CD4+ T cells, little is known about the functions and pro-/anti-tumour properties of $\gamma\delta$ T cells.

$\gamma\delta$ T cells are ‘unconventional’ T cells with innate and adaptive features that represent a relatively small subset of T cells overall²⁸⁸. They show tissue-specific localisation of subpopulations and are classified into three subtypes based on the expression of δ chains: V δ 1 (enriched in mucosal and epithelial tissues such as the gastrointestinal tract), V δ 2 (mainly found in peripheral blood) and V δ 3 (distributed mainly in the liver)²⁸⁹. These tissue resident $\gamma\delta$ T cells display broad functional plasticity and play an important role in tissue homeostasis and cancer immunosurveillance¹⁶⁹. Activation of $\gamma\delta$ T cells involves direct cytotoxicity of target cells as well as the production and release of cytotoxic granules and chemokines²⁸⁸. Furthermore, because of their

broad non-MHC-restricted antigenic specificity and potent cytotoxicity, they represent attractive alternative targets for immunotherapy.

Although $\gamma\delta$ T cells were identified as the strongest predictor of outcome across several solid organ cancers, this finding did not extend to CRC. Furthermore, these analyses did not distinguish between $\gamma\delta$ T cell subsets. Both pro-tumour and anti-tumour effects have been observed in CRC^{290,291}. Deciphering the phenotype and functions of $\gamma\delta$ T cells in CRC is important not only for prognostic purposes, but also to aid the design of novel immunotherapeutic strategies. The aim of this study was to evaluate the functional properties of $\gamma\delta$ T cell subsets in treatment naïve non-metastatic CRC using flow cytometry and single cell RNA sequencing.

8.2 Methods

8.2.1 Study population

A consecutive series of patients with histologically confirmed, non-metastatic, treatment naïve colon cancer undergoing surgery with curative intent in St. Vincent's University Hospital were prospectively studied. The study protocol was reviewed and approved by the institutional Research and Ethics Board. All samples were obtained with written informed consent.

Exclusion criteria included stage IV disease, neoadjuvant chemotherapy or radiotherapy, emergency presentation, or pre-existing immunosuppression secondary to autoimmune disorders or medication. Clinical staging was according to the 8th Edition of the American Joint Committee on Cancer tumour node metastasis (TNM) staging system and based on computed tomography (CT) of the thorax, abdomen and pelvis.

8.2.2 Pathological analysis

Haematoxylin and eosin sections of the resected specimen were analysed using a minimum dataset and a standardized reporting system previously described.

8.2.3 Immunophenotyping

Immunophenotyping was performed by single cell RNA sequencing and flow cytometry. Sample collection, storage and processing was as outlined in Methods 2.7.3-4. In brief, single-cell suspensions of

tumour and healthy colonic tissue were obtained. A standardized digestion protocol of DNase 1 and collagenase IV was used. For intra-cytoplasmic cytokine staining, cells were stimulated *in vitro* with a standardized stimulation cocktail of PMA, ionomycin, and Brefeldin A for 4 hours at 37 °C. Dead cells were excluded by live/dead staining using Zombie Aqua (BioLegend). Following extracellular staining, cells were fixed and permeabilized using the eBioscience Transcription Factor Staining Buffer Set. Once fixation and permeabilisation was complete, intracellular staining was performed. All data were collected on a BD Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star). Single cell RNA sequencing was performed on 5 colon tumours using the 10x genomics platform on isolated subpopulations of T cells as described in Methods 2.6.

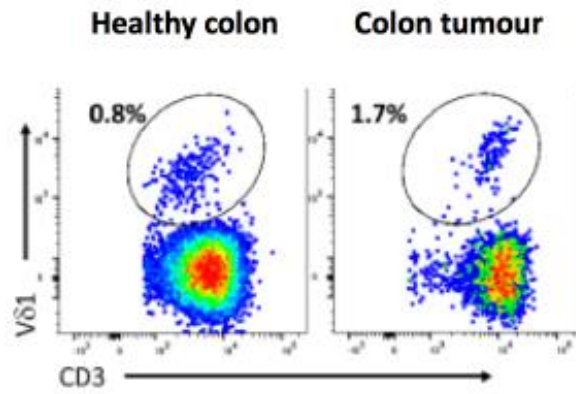
8.2.4 Statistical analysis

Data are expressed as standard error of the mean (SEM). The dot plots represent each individual patient sample. Wilcoxon matched pairs signed rank test was used for statistical analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Analyses were performed with Prism (GraphPad Prism version 7.0).

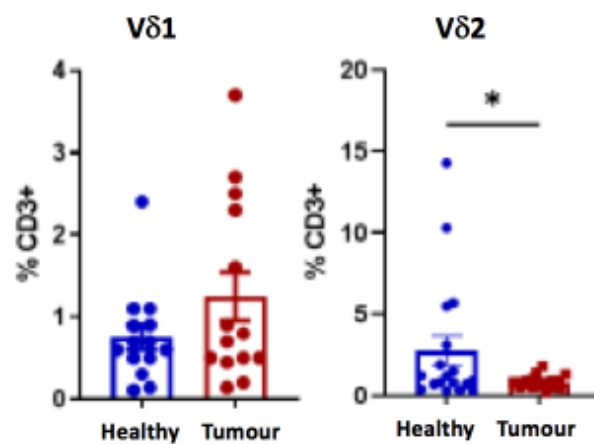
8.3 Results

8.3.1 Enrichment of V δ 1 cells in tumour compared to healthy colon

The V δ 1 and V δ 2 subsets were evaluated in tumour and healthy uninvolved colon. Analysis by flow cytometry revealed a significant reduction in the proportion of V δ 2 cells and enrichment of V δ 1 cells in tumour compared to healthy uninvolved colonic tissue (*Figure 37*). The V δ 1 subset became the focus of the remainder of the study. The hypothesis was that the phenotype of tumour-infiltrating V δ 1T cells may contribute to the heterogeneity in disease outcome observed in colon cancer compared to other solid organ cancers. To investigate this hypothesis, we performed single cell RNA sequencing in addition to further flow cytometry. Similar proportions of $\gamma\delta$ T cells, MAIT cells, NK cells, Tregs and adaptive CD4 & CD8 T cells were sequenced from tumour samples. Clusters were identified on the basis of differential gene expression and expression of lineage specific genes. $\gamma\delta$ T cells differed from conventional T cells in their effector, checkpoint and activatory receptor expression patterns, and were more similar to NK cells than conventional T cells in gene expression.



A.



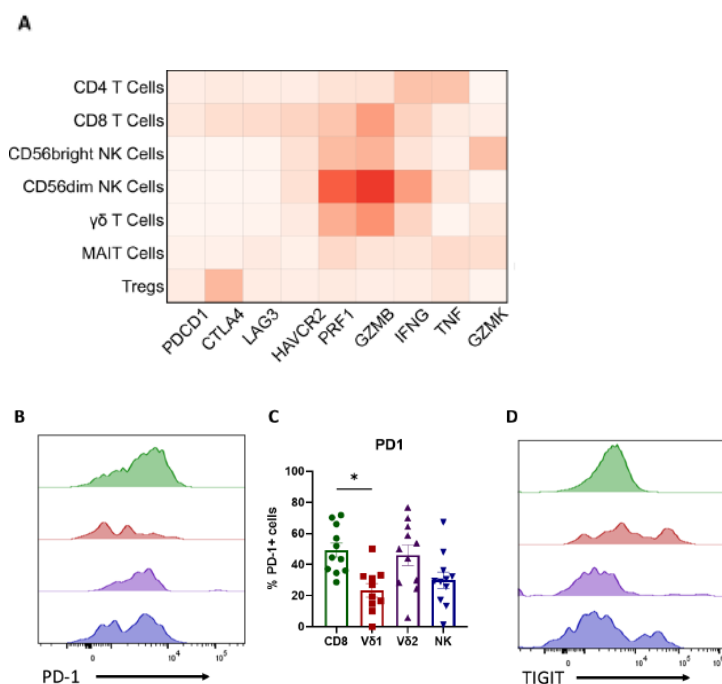
B.

Figure 37. (A) Representative FACS plots of V δ 1 T cells in healthy colon and colon tumours. (B) Percentage of V δ 1 & V δ 2 T cells in healthy colon and colon tumours.

8.3.2 Inhibitory checkpoint expression and cytotoxicity of intra-tumoral $\gamma\delta$ T cells

Single cell RNA sequencing data sets were used to evaluate immune checkpoint, effector molecule and cytotoxicity receptor expression (Figure 38A). Low levels of PD1 (*PDCD1*), CTLA4, TIM3 (*HAVCR2*) and LAG3 expression on $\gamma\delta$ T cells were observed, similar to NK cells and lower than conventional T cells (Figure 38A). High levels of

cytotoxic effector molecules, granzyme B (*GZMB*), perforin (*PRF1*), and interferon gamma (*IFN* γ) were found, similar to NK cells and CD8 T cells (*Figure 38A*). Therefore, $\gamma\delta$ T cells have a cytotoxic phenotype without typical inhibitory markers in comparison to other T cells (CD8, CD4, MAIT cells). These findings were confirmed by flow cytometry in an independent cohort of patients with colon cancer. V δ 1 T cells showed the lowest levels of PD1 expression (*Figure 38B-C*) but expressed high levels of TIGIT (*Figure 38D-E*), an inhibitory receptor previously described on NK cells. LAG3 and TIM3 expression was significantly lower in V δ 1 T cells compared to CD8 T cells (*Figure 38F-G*). Compared to other cytotoxic lymphocyte populations, V δ 1 T cells expressed similar, and in some cases higher levels of GZMB, GZMK, *IFN* γ and TNF α (*Figure 38H-K*). Thus, $\gamma\delta$ T cells in tumour are a cytotoxic T cell subset with potentially less inhibition than other T cell populations (CD8, CD4, MAIT cells).



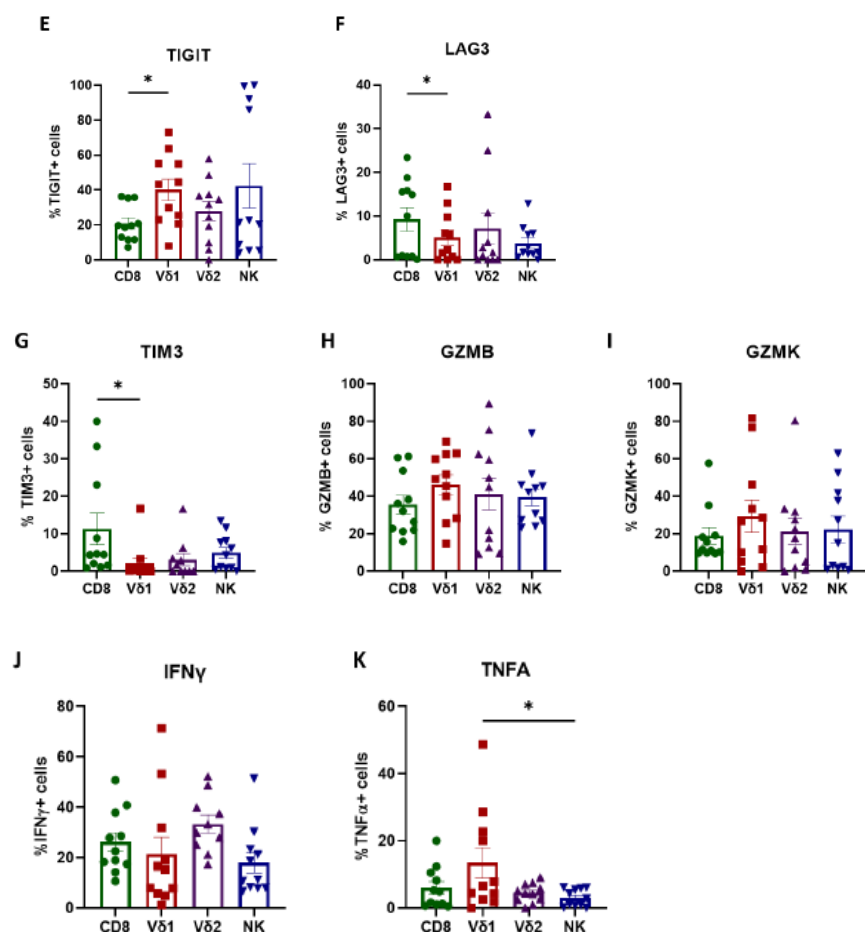


Figure 38. (A) Heatmap showing gene expression of immune checkpoints in cell subsets in colon tumours. (B) Representative FACS plots of PD-1 expression in CD8, Vδ1, Vδ2 and NK cells from colon tumours. (C) Percentage of CD8, Vδ1, Vδ2 and NK cells positive for PD-1. (D) Representative FACS plots of TIGIT expression in CD8, Vδ1, Vδ2 and NK cells from colon tumours. (E) Percentage of CD8, Vδ1, Vδ2 and NK cells positive for TIGIT. (F-G) Percentage of cell subsets expressing immune checkpoints LAG3 & TIM3. (H-K) Percentage of cell subsets expressing effector molecules GZMB, GZMK, IFN γ & TNF α . Data presented as mean $\pm$ SEM. Data were analyzed using Friedman test, with Dunn's multiple comparison test. (F-K n=11, * p<0.05).

8.3.3 Functional heterogeneity of intra-tumoral $\gamma\delta$ T cells

Three functionally distinct clusters of $\gamma\delta$ T cells were identified: a cytotoxic cluster (purple), a PLZF⁺ wound-healing cluster (blue) expressing *AREG* and an intermediate cluster expressing genes (*KRLB1*, *CXCR4*, *FOSB*, *JUNB*) associated with tissue residence (red) but also effector molecules (*Figure 39A*). Markers of exhaustion were mainly expressed by the cytotoxic cluster, suggesting that the other two clusters were not exhausted but rather a functionally distinct subset (*Figure 39B*). The PLZF⁺ cluster expressed the lowest level of IFN γ but was significantly enriched for *AREG* (*Figure 39C*). The intermediate cluster expressed *AREG* but also IFN γ (*Figure 39C*).

Tumour-infiltrating $\gamma\delta$ T cell *AREG* production was then assessed by flow cytometry. The cells were stimulated with PMA. *AREG* was produced by V δ 1 and V δ 3 subsets, which are typically tissue resident, while the V δ 2 subset produced IFN γ (*Figure 39D-G*). A large proportion of V δ 1 cells however, also produced IFN γ , with some also producing both *AREG*⁺ and IFN γ ⁺ cells, possibly representing the ‘intermediate’ cluster. Thus, V δ 1 T cells are heterogeneous in colon cancer and consist of populations that can produce IFN γ or *AREG*.

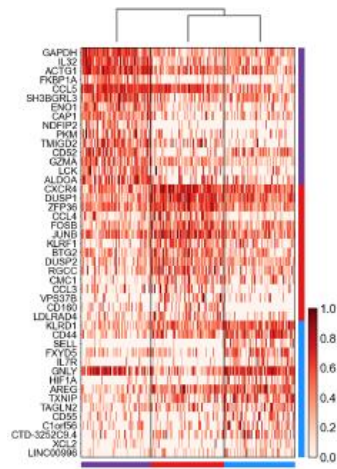
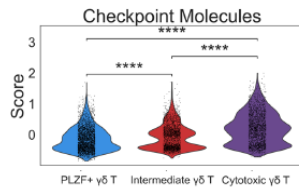
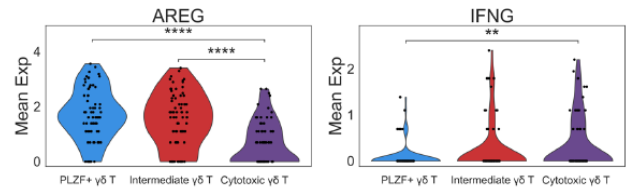
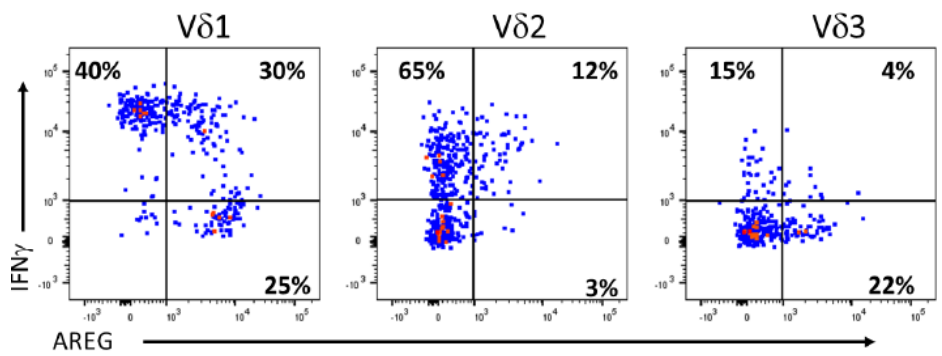
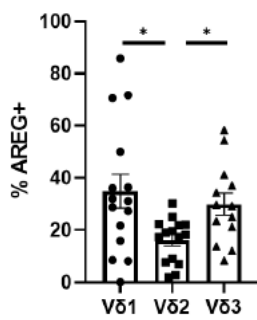
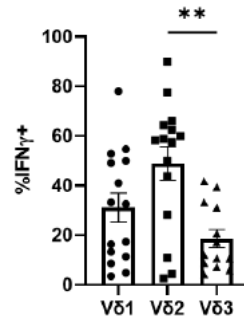
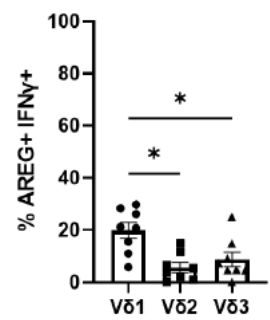
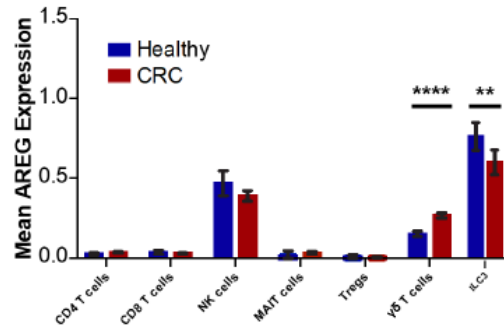
A**B****C****D****E****F****G**

Figure 39. (A) Heatmap showing the top 15 differentially expressed genes between $\gamma\delta$ T cell clusters in colon cancer. (B) Z-score of expression of immune checkpoints was calculated and plotted for $\gamma\delta$ T cell clusters from colon cancer. (C) Gene expression of *AREG* and *IFN γ* in $\gamma\delta$ subsets from scRNAseq of colon tumours. (D) Representative FACS plots of *IFN γ* and *AREG* expression in V δ 1, V δ 2 & V δ 3 T cells. (E) Percentage of *AREG*⁺, (F) *IFN γ* ⁺ and (G) *AREG*⁺*IFN γ* ⁺ V δ 1, V δ 2 & V δ 3 T cells. Data presented as mean $\pm$ SEM. Data were analyzed using Friedman test, with Dunn's multiple comparison test (G n=15, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

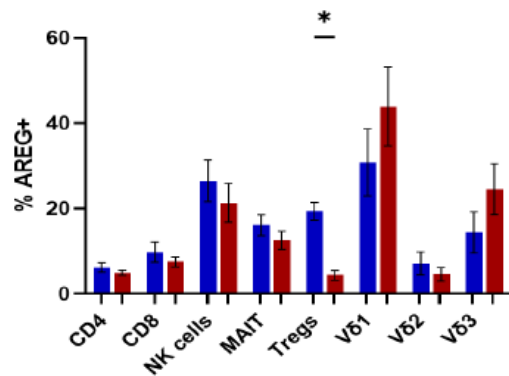
8.3.4 $\gamma\delta$ T cell expression of *AREG* in healthy colon

Single cell RNAseq was also performed on matched tumour and healthy colon samples. In both tumour and healthy colon, *AREG* gene expression was associated with innate cells, $\gamma\delta$ T cells, NK cells and ILC3s. Interestingly, $\gamma\delta$ T cells were the only population that exhibited increased *AREG* expression in the tumour compared to healthy colon (*Figure 40A*). V δ 1 T cells also displayed the highest levels of *AREG* expression in tumour (*Figure 40B*). *IFN γ* expression was seen in several innate and adaptive cell types, and was significantly increased in $\gamma\delta$ T cells in the tumour (*Figure 40C*).

A



B



C

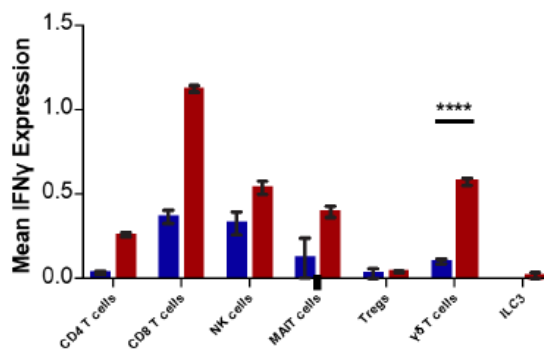


Figure 40. (A) Proportion of immune subsets expressing AREG in normal colon (blue) and colon tumour (red). (B) Flow cytometry analysis of AREG in immune subsets in healthy colon (blue) and colon tumour (red). (C) Proportion of immune subsets expressing IFN γ in normal colon (blue) and colon tumour (red).

8.3.5 AREG expression is associated with NKp80+ Vδ1 cells

The next step was to identify surface receptors associated with the wound healing and cytotoxic $\gamma\delta$ T cell phenotypes. Several inhibitory checkpoints and cytotoxicity receptors were evaluated to identify potential immune signatures associated with AREG or IFN γ production in Vδ1 T cells. NKp80 (*KLRF1*) was strongly associated with AREG expression, while TIGIT was associated with a cytotoxic phenotype (*Figure 41A-B*). Both NKp80 and TIGIT were most highly expressed on Vδ1 T cells compared to Vδ2 (*Figure 41C*). The production of AREG and IFN γ in four Vδ1 subsets was evaluated using TIGIT and NKp80 co-expression, (TIGIT-NKp80-, TIGIT+NKp80-, TIGIT+NKp80+, TIGIT-NKp80+). TIGIT+NKp80- cells had the highest levels of IFN γ production and the lowest levels of AREG production in colon tumours. Inversely, NKp80+TIGIT- cells had the highest levels of AREG production and the lowest level of IFN γ (*Figure 41D-E*).

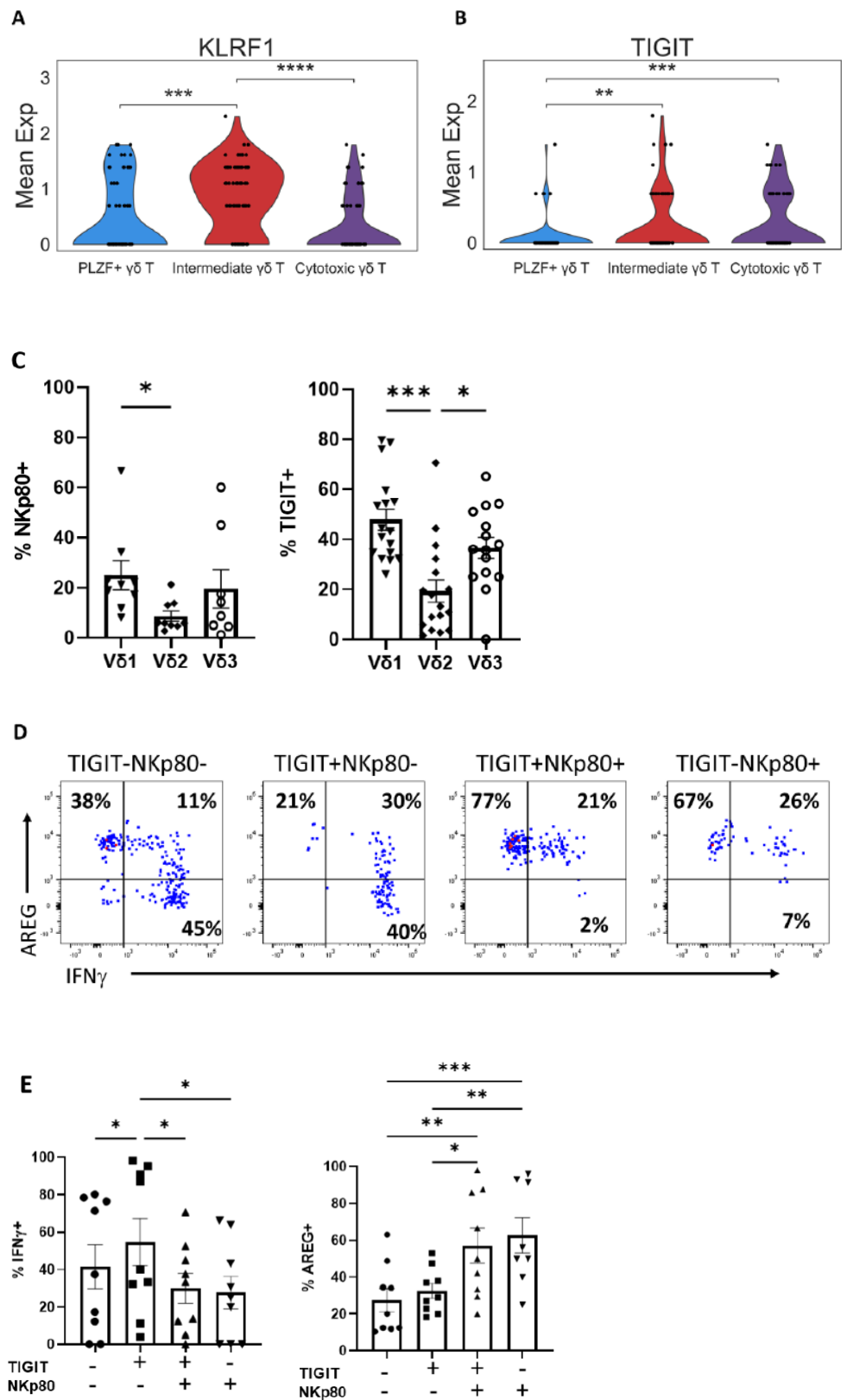


Figure 41. (A-B) Gene expression of *KLRF1* & *TIGIT* in $\gamma\delta$ T cell subsets in colon tumours. (C) Percentage expression of NKp80 & TIGIT in V δ 1, V δ 2 & V δ 3 T cells in colon tumours. (D) Representative FACS plot of AREG & IFN γ expression in V δ 1 T

cells subsetted by NKp80 & TIGIT expression after 4hr PMA stimulation. (E) Percentage of IFN γ & AREG expression in V δ 1 T cells subsetted by NKp80 & TIGIT expression.

8.4 Discussion

The tumour ecosystem is composed of many subsets of lymphocytes, with considerable variation between immune cell types and cancer-specific outcomes observed. $\gamma\delta$ T cells are a small but important component of this microenvironment. They play a substantial role in anti-tumour immunity, and represent an important prognostic indicator of clinical outcome. They also represent attractive targets for immunotherapy. The majority of data on the biology of human $\gamma\delta$ T cells comes from blood-circulating cells, which are mainly V δ 2. This study evaluated the biology of human $\gamma\delta$ T cells in colonic tumours and in healthy colon.

In the present study, scRNA seq data and flow cytometry were used to identify two functionally distinct subsets of $\gamma\delta$ T cells in human colon tumours. One subset displayed an AREG-producing phenotype, whilst the other exhibited high cytotoxicity with low checkpoint expression. Interestingly, the presence of these two opposing $\gamma\delta$ T cell subsets depends on the tumour type, with the AREG subset only identified in colon cancer.

AREG is an epidermal growth factor receptor (EGFR) ligand which is commonly overexpressed in CRC²⁹². The AREG/EGFR signaling pathway regulates key cellular events that contribute to tumour proliferation, migration, invasion, and angiogenesis²⁹³. Mutations, and protein overexpression of various components of this pathway not only contribute to tumorigenesis but also impact prognosis and

provide specific targets for therapeutic intervention²⁹⁴. High levels are associated with favorable response to anti-EGFR therapy²⁹⁵. AREG is typically associated with MSS tumours, a wound healing molecular phenotype, (CMS2) and worse oncological outcomes. It is plausible that in MSS CRC, V δ 1 T cells provide AREG which facilitates tumour growth, and at the same time fail to provide an early source of IFN γ to promote a robust adaptive response. Thus, the TME could potentially be altered to allow immune activation. Depleting AREG-producing V δ 1 T cells could represent a novel immunotherapeutic approach for MSS tumours

AREG is also produced by $\gamma\delta$ T cells in healthy uninvolved colon. They maintain an intermediate phenotype important for tissue homeostasis, with the potential to maintain and repair tissue integrity with an AREG-based response, yet also to ignite an IFN γ -based response to tumours. In the setting of cancer, $\gamma\delta$ T cells upregulated AREG production. It is plausible that the tumour is recognized as a 'wound' leading to upregulation of AREG, prevention of an appropriate immune response, and a perpetuating circumstance in which the 'wound' does not heal. Reconfiguration of the $\gamma\delta$ T cell population has also been observed in chronic gastrointestinal inflammation. Mayassi et al. found loss of AREG producing $\gamma\delta$ T cells and expansion of IFN γ producing $\gamma\delta$ T cells in the setting of coeliac disease²⁹⁶.

The identification of these distinct functional V δ 1 subsets by surface markers may be beneficial when designing novel therapies. In the present study, cytotoxic V δ 1 cells were identifiable by the expression of TIGIT (a NK cell related inhibitory receptor)²⁹⁷ These data would suggest that current checkpoint therapies do not have significant effects on $\gamma\delta$ T cells in human colon tumours, as they lack expression of PD-1 or CTLA-4. TIGIT however, is an important checkpoint for innate T cells and NK cells, and may provide an important target for combination therapies using $\gamma\delta$ T and NK cells to boost anti-tumour immunity^{275,298,299}. NKp80 expression was associated with AREG production and represented a marker of pro-wound healing $\gamma\delta$ T cells. NKp80 has previously been shown to be differentially expressed in human V δ 1 and V δ 2 T cells³⁰⁰, however in NK and CD8 T cells its expression is associated with cytotoxicity and an effector memory phenotype respectively^{301,302}.

Due to their functional plasticity, $\gamma\delta$ T cells are susceptible to polarization by a particular environment. In response to certain cytokines which are elevated in the tumour microenvironment, these cells can adopt pro-tumour functions¹⁶⁸. For example, although human $\gamma\delta$ T cells rarely produce IL-17, these cells have been found to produce IL-17 in the setting of colorectal cancer. Breach of the gut epithelial barrier, resulting in the activation of IL-23 producing dendritic cells, led to $\gamma\delta$ T17 polarisation of V δ 1 cells²⁹⁰. In the present study we have shown that, despite their strong association with positive prognosis in multiple solid organ malignancies, $\gamma\delta$ T cells can

be skewed toward an AREG-producing pro-tumour phenotype in colon cancer. Enhancing the proportion of cytotoxic V δ 1 $\gamma\delta$ T cells and depleting the proportion of wound healing V δ 1 $\gamma\delta$ T cells may represent a novel immunotherapeutic strategy.

Chapter 9. Discussion and Conclusion

9.1 Discussion

9.1.1 Summary of key findings

Immunopathological features of early-onset colorectal cancer

EOCRC accounts for one in ten CRC diagnoses, representing the second most common cancer and the third leading cause of cancer-related death in adults aged less than 50. Clinicopathological and survival data specific to EOCRC are lacking, and the sensitivity of the disease to conventional therapeutic strategies is unknown. In this thesis, data on patients with EOCRC was collected at local and international level. Clinical characteristics, molecular features and oncological outcomes were analysed.

From the series of studies described, a number of key observations can be made. As the volume of data increases, clinical and pathological patterns are emerging. For reasons unknown, the increasing incidence of EOCRC is predominantly driven by a rising rate of rectal cancer. The study detailed in Chapter 3 compared disease specific outcomes of patients diagnosed with non-metastatic rectal cancer (undergoing surgery with curative intent) aged less than 50 years to those aged over 50 years. Young patients were more likely to have tumours with MSI, be diagnosed of Lynch syndrome, and receive both neoadjuvant and adjuvant therapy. Despite accessing more treatment, there were no significant differences in 5-year disease

free survival or in recurrence rates.

Similar survival despite more treatment raises a number of questions. Firstly, do young patients require more therapy because of aggressive or treatment resistant tumour biology? EOCRC is thought to represent an aggressive disease as the majority of patients present with advanced stage (III or IV) and frequently display unfavorable histopathological features. Not only are young patients more likely to receive neoadjuvant and adjuvant therapy, they also more commonly receive multi-agent regimes. Secondly, how does EOCRC respond to conventional therapies? It is possible that differences in biomolecular profile influence the response of EOCRC to treatment. A uniquely targetable profile however, has not yet been identified. Studies comparing early and late onset disease have found overlapping molecular processes with some differences. The implication of these differences on treatment response is unclear. Importantly, in the absence of a definitive oncological benefit, care must be taken to avoid overtreatment of low risk disease, particularly given the significant toxicity and long-term morbidity associated with treatment e.g. bowel/bladder/sexual dysfunction and infertility.

Although valuable and important to define unmet research needs, individual institutional data alone is of limited value in addressing the key questions surrounding EOCRC. To overcome the challenge of small patient numbers and collect large volume data, the international

Research in Early Age Colorectal Cancer Trends (REACCT)

collaborative group was established. Over 130 institutions in 43 countries joined the collaborative. The study detailed in *Chapter 4* analysed clinicopathological features and oncological outcomes of 3378 patients diagnosed with non-metastatic EOCRC.

Tumours of the sigmoid colon and rectum accounted for 70.1%. Five-year disease-free survival for stage I, II, and III colon cancer was 96%, 91%, and 68% respectively. The equivalent rates for patients with rectal cancer were 91%, 81%, and 62%. Pathological stage III disease was diagnosed in 43% patients with colon cancer and in 41.7% patients with rectal cancer. Microsatellite instability was present in 20.0% (1 in 4 colon cancers and 1 in 10 rectal cancers). Approximately one third of patients with MSI tumours (colonic and rectal) were diagnosed with a definable hereditary cancer syndrome. Importantly, 35.2% of those with MSI colon cancer and 30.4% of those with MSI rectal cancer, had not had genetic testing at the time of data collection.

Prognostication and therapeutic decision making in CRC is largely based on histopathological analysis of the resected specimen and the TNM staging system. Variable outcome however occurs among patients with the same disease stage, presumably in part related to tumoral molecular features. MSI is identified in approximately 15% of CRCs whilst the remaining 85% demonstrate microsatellite stability (MSS). The dichotomisation of CRC into MSI and MSS has important

prognostic and therapeutic implications. The study detailed in *Chapter 5* compared the clinical characteristics, pathological features and disease specific outcomes of early onset MSI and MSS colon cancers.

Early age MSI colon cancer frequently occurs in patients with a family history of CRC, it is typically located in the proximal colon, and is often morphologically poorly differentiated/signet ring. Unlike in older age groups, MSI in young patients is not associated with female preponderance, and is associated with equal rates of nodal positivity and tumour budding as MSS tumours. In terms of survival, patients with MSI cancer had better disease-specific survival in all disease stages although these differences were not statistically significant. The survival advantage observed however, is clinically significant, in particular for stage III disease. Several similarities and differences exist between young and older-onset MSI colon cancer (summarised in *Figure 42*)

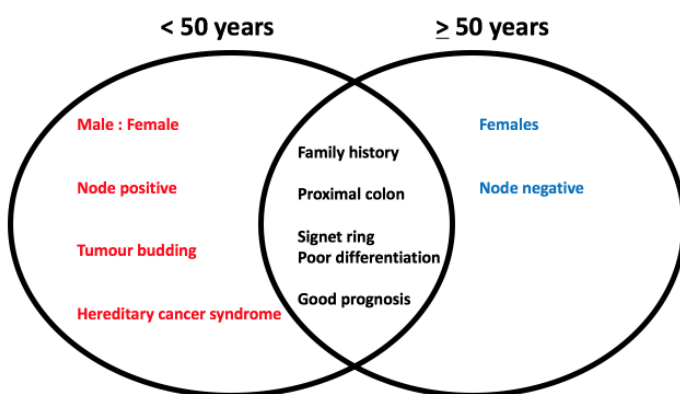


Figure 42. Age-based comparison of MSI colon cancer.

Outcomes of patients with early age onset rectal cancer according to MSI status were evaluated in the study described in *Chapter 6*. In this study of 400 patients with early age onset rectal cancer, 12.5% of patients demonstrated MSI. MSI was associated with reduced likelihood of nodal positivity, increased rate of pCR and improved disease-specific survival (Table 19). The increased pCR rate in young patients with MSI rectal cancer is particularly interesting as it may open the possibility of organ preservation in this specific group. Socioeconomic, psychosocial and quality of life factors are arguably extremely important in these otherwise healthy individuals. The negative impact of major pelvic surgery on lower gastrointestinal, genitourinary and sexual function can be significant. Total neoadjuvant therapy or even immunotherapy may represent promising treatment strategies. A small (n=12) prospective phase 2 trial evaluating dostarlimab (an anti-PD-1 monoclonal antibody) in patients with MSI rectal cancer reported a cCR of 100%³⁰³. Importantly, young patients require a model of care that is not only disease-focused but encompasses an individualised multidisciplinary approach.

	<50	>50
Sex	Female > Male	Male = Female
Nodal positivity	MSI < MSS	MSI = MSS
pCR	MSI > MSS	MSI = MSS
Survival	MSI > MSS	MSI = MSS
Hereditary cancer syndrome	MSI > MSS	MSI > MSS

Table 19. Age-based comparison of MSI rectal cancer.

The immune landscape of CRC

In spite of advances in systemic neoadjuvant and adjuvant therapy, alternative therapeutic mechanisms to improve disease control are needed. Immunotherapy has emerged as an attractive strategy. Intra-tumoral immune landscape is an important predictor of disease-related outcome in CRC and is influenced by tumour biology. The studies in outlined in Chapters 7 and 8 aim to evaluate the intra-tumoral immune profile of CRC. As EOCRC is relatively rare, no age restriction was applied to these studies in order to accrue enough samples. Patients aged 18 or over who fulfilled the inclusion criteria were included.

The study in described in Chapter 7 evaluated inhibitory checkpoint expression on tumour-infiltrating lymphocytes in treatment naïve non-metastatic CRC according to MSI status. Flow cytometry analysis found that expression of inhibitory markers PD-1 and TIGIT were significantly increased on CD3⁺ cells (T cells) in the tumour compared to uninvolved healthy colonic tissue among the entire study group, indicating T cell recognition of tumour. Expression of PD-1, was significantly higher in MSI tumours compared to MSS, however, one in four MSS tumours had comparable levels of PD-1 expression to the MSI group. TIGIT expression did not differ between the MSI and MSS groups. On single cell analysis of MSS tumours, inhibitory checkpoint expression was predominantly observed on CD8⁺ T cells, with a range of checkpoints expressed (PD-1, TIGIT, TIM-3, LAG3). Functional status assessing intra-tumoral CD3⁺ cell production of IFN γ , IL-17a, and amphiregulin did not differ significantly between groups. Overall,

these data demonstrated that TIL inhibitory checkpoint expression and production of pro- and anti-tumour immune mediators, vary significantly among patients with the same microsatellite status. Thus microsatellite status in isolation may not accurately predict response to immunotherapy.

The tumour microenvironment consists of many types of immune cell populations that display different functional properties. Building on the data presented in Chapter 7, the study described in Chapter 8 evaluated the functional properties of $\gamma\delta$ T cell subsets in colon cancer. A subset of $\gamma\delta$ T cells (V δ 1) were found to be enriched in tumour compared to healthy colonic tissue. Furthermore, functionally distinct subpopulations of V δ 1 cells were identified in the tumour. One subset produced IFN γ , demonstrating a cytotoxic phenotype. The other subset demonstrated a wound healing phenotype, producing amphiregulin (AREG), which can promote tumour growth and contribute to an immunosuppressed tumour microenvironment). The surface marker NKp80 (*KLRF1*) was strongly associated with AREG expression, while TIGIT was associated with the cytotoxic phenotype. Increasing the proportion of cytotoxic V δ 1 $\gamma\delta$ T cells and reducing the proportion of wound healing V δ 1 $\gamma\delta$ T cells represents a novel immunotherapeutic strategy in colorectal cancer.

9.1.2 Limitations

A number of limitations are acknowledged.

Protocol limitations

Retrospective nature

The relative rarity of early age onset colorectal cancer limits the feasibility of prospective studies on the subject over a short period of time. The time needed to collect enough data for a meaningful analysis would be significant. Thus the clinical studies described in this thesis are retrospective. Specific limitation of these retrospective studies include selection bias and the lack of complete data set for the entire patient cohort.

Sample size

The sample size of a study influences the accuracy of the findings and the power to draw conclusions. A limitation of the study outlined in Chapter 3 is the relatively small sample size which limits the generalizability and external validity of the results. Single institutional data relating to an uncommon disease process are too small to draw meaningful conclusions. Nonetheless, the data represents the experience of a high volume tertiary referral centre. Notably, the findings raised a number of important questions and identified unmet research needs in early age onset rectal cancer, providing a platform for the larger studies described in Chapters 4, 5 and 6.

Similarly, sample size was also a limitation of the prospective studies outlined in Chapters 7 and 8. This was partly related to the strict inclusion criteria however, the most significant challenge to accruing patients was the COVID-19 pandemic. For a significant proportion of the investigative period of this doctoral study, COVID-19 related restrictions were in place. Elective surgeries were cancelled for a period of time. Even when operating recommenced, samples could not be collected as it was not morally or professionally ethical to expose patients undergoing cancer surgery to unnecessary non-clinical team members and increase their risk of contracting COVID-19.

Collaborative data

In order to obtain a large sample of patients, the REACCT Collaborative was established. Large datasets can be used to efficiently answer multiple research questions. The data collected in this thesis represents patients from all over the world. Demographics, socioeconomic status, environmental exposures, population-based preventative/screening strategies, access to diagnostics and treatment differ worldwide. As a result, the findings are representative of wider populations, and the results are generalizable and relevant to more patients. Nonetheless, several limitations of data collected via a global collaborative network are acknowledged. Firstly, heterogeneity in treatment protocols (e.g. indications for chemotherapy, chemotherapeutic agents administered) as well as pathological/molecular assessment existed across the collaborative group. Histopathological and molecular evaluation of the resected

specimen varied among institutions. As such parts of the pathological and molecular dataset were incomplete. In addition, methods for testing MSI differed among centres with some using PCR whilst others used IHC to identify loss of MMR proteins and therefore indirectly MSI. Another limitation of large multi-institutional datasets, is that it is not possible to independently verify the data, and the accuracy and quality of data collection in the participating institutions must be relied upon. Nonetheless, the data collected via the collaborative group represents important ‘real-world’ data and provides a useful snap shot from which to design future prospective trials.

Time interval

As EOCRC is a relatively rare phenomenon and a large sample size is preferable when evaluating clinicopathological features and oncological outcomes, the study interval extended from 2000 to 2021 in order to capture as many patients as possible. A limitation of a long study interval is that chemotherapy and radiotherapy strategies have evolved over that time period meaning patients may have received different treatments depending on the time of diagnosis. This may limit the applicability of the results. Furthermore, reflex MSI testing and genetic testing where appropriate were not common practice in many institutions for part of the study period. As a result, MSI status was not known in a significant proportion of patients. Similarly, genetic testing had not been performed on all patients with MSI. Thus

it is not possible to accurately estimate the rate of MSI or hereditary cancer syndrome.

Statistical analysis

The Kaplan-Meier method was used to estimate the probability of survival beyond a certain time point (1, 3 and 5 years). Kaplan-Meier curves were generated to graphically illustrate the estimated survival function by plotting estimated survival probabilities against time. A limitation of survival analyses is censoring. This occurs when patients are lost to follow-up leading to incompletely observed survival times. As a result, censored patients are included in estimates of survival probabilities at time points preceding their censoring time point, and excluded from the analysis thereafter. This is known as right censoring.

In the studies described in Chapters 3 and 5 the log rank test was used to compare survival of two groups. It tests the null hypothesis that there is no difference in the probability of an event (death or disease recurrence) occurring at any time point. The power of the log rank test depends on the number of observed events rather than the sample sizes.

A limitation of the Kaplan-Meier method of survival analysis is that the log rank test is purely a significance test. It only generates a p-value but cannot estimate the size of the difference between the

groups. Furthermore, it does not adjust for covariates that might impact survival.

In Chapter 5, patients with MSI colon cancer demonstrated better survival than those with MSS colon cancer across all disease stages. The difference greatest was in patients with stage III disease. These differences however, were not statistically significant (although likely to be clinically significant). Lack of statistical significance may be due to inadequate sample size and statistical power. It is possible that a type two error has occurred where the null hypothesis (that no statistically significant difference exists) has been incorrectly rejected. A larger sample size would reduce the risk of a type two error. In Chapter 6, patients with MSI rectal cancer also demonstrated better survival than those with MSS rectal cancer across all disease stages. It was decided not to perform the log rank test in this study, as the sample size was smaller than the sample size of the study in Chapter 5 and so statistical analyses of survival data could be misleading and the conclusions may be unreliable.

Technical limitations

Flow cytometry

Flow cytometry is one of the most commonly used techniques to characterise immune cells in solid tumours. Several limitations are acknowledged.

Sample transfer, storage and processing

Ex vivo analysis of tumour and colonic tissue can result in alterations to cellular structure during sample transfer, storage and processing.

Samples were placed in sterile prepared HBSS media, placed on ice and transferred to the laboratory immediately for storage. HBSS is an isotonic solution which maintains a neutral pH. Although it provides cells with salts to maintain osmolarity, to preserve cells in their natural state (i.e. prevent bursting), a degree of cell loss during transfer is inevitable.

Once in the laboratory, tissue samples were stored in Cryostor media at -80°C. Cryopreservation is a process whereby cells are cooled to very low temperatures in order to halt cellular metabolism and preserve cellular structure. A limitation of cryopreservation is that it can affect cell viability. During freezing, cells can be damaged by the formation of intracellular ice-crystals and osmotic imbalance between the intra-and extra-cellular space leading to dehydration and shrinkage³⁰⁴. Several studies have reported immune cell viability of between 60-90% after cryopreservation and thawing, along with preservation of cytokine production and expression of surface markers^{305,306}.

In order to minimize cell loss during the thawing process, the cells were carefully thawed by gently swirling the cryovials in a 37°C water bath until the edge of the frozen cells detached from the side of the cryovial. The frozen cells were then poured into the warm thawing

medium and centrifuged. Despite these measures, a degree of cell loss during the storage and thawing process is inevitable. Samples were analysed fresh and following cryopreservation to assess for differences in cell viability between the two techniques and to evaluate the feasibility of cryopreservation. Acceptable immune cell survival was observed with cryopreservation and so this technique was used for the experiments presented in this thesis. In order to be analysed by the flow cytometer cells must exist in a single-cell suspension. Enzymes are used to digest solid tumours. These dissociation techniques can also impact cell viability and disrupt natural cellular and tissue architectures.

Panel design

Another important limitation of flow cytometry is the lack of standardization in antigen markers, fluorescence, and antibody combinations. Designing the optimal panel to generate the most accurate results can be challenging, particularly when using multicolour flow cytometry. Several steps were taken to optimize the panel design. Colours with the best separation between positive and negative populations (bright colours) were used for markers that were anticipated to be more difficult to detect due to low expression or poor antibody affinity and vice versa for dim colours. Where multiple clones for the same antibody were available several were evaluated. Once designed the panel was tested on PBMCs to identify any issues and optimize accordingly. In addition to problems with panel design, technical errors during staining, spectral overlap, inter-experimental

variability, and subjective data interpretation may also limit the accuracy of the data.

T cell location

Another limitation of flow cytometry is the inability to determine the location of immune cells in the tumour i.e. in the tumour core or at the invasive margin. Knowledge of T cell location may be useful in determining whether these cells are successfully infiltrating the tumour or being halted at the periphery. T cell location is also required to calculate the Immunoscore. It had been planned to determine the location of TILs within the tumour (core vs invasive margin), and to compare infiltration pattern of MSI and MSS tumours by histological analysis of paraffin embedded tumour samples (via immunohistochemistry) in St Vincent's University hospital. Due to the COVID-19 pandemic, it was not possible to do this as laboratory resources for research were re-allocated to COVID-19 testing. Transfer of samples to the laboratory in Trinity College Dublin was not an option due to GDPR.

9.1.3 Future perspectives

The oncotherapeutic sensitivity of EOCRC

The key question in EOCRC is whether tumour biology differs to that of late-onset disease. Although some differences have been observed, a unique biomolecular profile that could potentially be treated with targeted therapy has not yet been identified. An integrated scientific and clinical approach is needed to define the optimum management of

these patients. Sequencing of tumours would decipher the underpinning molecular mechanisms, while prospective clinical trials with a focus on early age disease would evaluate the impact of therapy on oncological outcomes. Future trials should place increased emphasis on young patients, either in isolation or as part of a wider study with sub-group analysis.

The genetic component of EOCRC

The genetic component of EOCRC remains undefined. Prevalence estimates of genetic predisposition reported in the literature vary, with significant heterogeneity in criteria of assessment and gene panel used. The spectrum of genes implicated in EOCRC has expanded remarkably with the development of next-generation sequencing. Importantly, genetic testing for hereditary CRC now encompasses broad multigene panels compared to the phenotype-specific single-gene assessment historically performed. A key finding of the study described in Chapter 4 was the high proportion of patients in whom MSI status had not been assessed. Similarly, of those with MSI tumours, one third had not had genetic testing at the time of data collection. Assessment of MSI status for all CRCs, with genetic testing where appropriate, was not standard of care for the majority of the study period. Universal testing (which has since been recommended) will facilitate prospective clinical trials to better define the genetics of EOCRC. One of the clinical implications of identifying hereditary cancer syndromes is the potential for vaccine-mediated immunity and prevention of carcinogenesis. Preliminary results of

vaccines for patients with Lynch Syndrome are promising, with exciting developments on the horizon.

The prognostic and therapeutic role of MSI in EOCRC

The prognostic impact of MSI in EOCC was raised in the study described in *Chapter 5*. In contrast to late-onset colon cancer, MSI in young adults was not associated with reduced likelihood of nodal positivity compared to patients with MSS tumours. Interestingly, the proportion of patients in the MSI group with pathological node positive disease was higher than that reported in the literature for all age MSI colon cancers. Lymph node involvement in CRC is a well-defined negative prognostic indicator, but the complex interplay between immune cells and tumour cells in the lymph nodes is incompletely understood. It is possible that the presence of tumour cells in lymph nodes is a step in the pathway to distant metastasis, and that while in lymph nodes the tumour cells acquire traits giving them the ability to metastasize. Through various mechanisms the tumour cells may evade immune cell attack in the lymph nodes, even orchestrating a degree of synergism with the immune cells. Immunotherapy may represent a potential option to modulate this tolerance and stimulate the anti-tumour actions of immune cells in the lymph nodes.

Disease-free survival rates were better in patients with stage III MSI tumours compared to those with stage III MSS tumours. Another noteworthy finding was the comparable rates of tumour budding in

MSI and MSS colon cancers. This is in contrast to all age CRCs, where tumour budding is less common in MSI tumours. The numbers in the analyses in this thesis are too small to draw definite conclusions. These findings need to be validated prospectively and support further investigation of the prognostic role of tumour budding in early onset disease. A prospective multicentre collaborative trial with standardized pathological assessment of tumour budding would define its utility in clinical practice. Nonetheless, these findings again highlight the importance of deciphering the impact of tumour biology on the immune response.

The therapeutic role of MSI in EORC was raised in the study described in *Chapter 6*. Interestingly, a higher pCR rate was observed in the MSI group. The focus of modern trials in this patient group should be on neoadjuvant therapy. Increasing pathological response not only improves locoregional control but may also facilitate an organ preserving approach or less radical surgery with avoidance of a stoma in select patients. Total neoadjuvant therapy, which is associated with superior pCR rates, may be an attractive strategy given that high-dose chemotherapy is usually well tolerated in this patient group. It is likely the immune-mediated effects of neoadjuvant therapy play an important role in pathological response. Variations in pCR rates may even be related to differences in the host immune response. There is some data to suggest patients who achieve a pCR may have a higher risk of anastomotic leak³⁰⁷. Hypothetical reasons for this may be higher rates of fibrosis and thus poorer healing in the

setting of a pCR. Furthermore, immunotherapy with checkpoint blockade may represent a revolutionary treatment option for select patients. Immunotherapy may even eradicate the tumour entirely, obviating the need for surgery. The challenge will be how to identify those patients who will respond to checkpoint therapy. As shown in Chapter 7, PD-1 expression varies significantly in patients with MSI cancer. Future trials evaluating response should assess pre-treatment levels of CD3+ PD-1+ as a guide to predicting response.

Quality of life and survivorship in EOCRC

Functional status and Health-Related Quality of Life (HR-QL) are important aspects of cancer care. Multimodal therapy for EOCRC involves major abdominopelvic surgery (with/without a stoma), radiotherapy, and chemotherapy. Young patients often receive more systemic treatment as well as multi-agent regimes compared to older patients. The long-term anatomical and physiological consequences of these treatments are significant and can cause bowel, bladder, and sexual dysfunction. Patients with EOCRC pose unique challenges compared to their older counterparts, and require different support measures during treatment but also in survivorship. Age at diagnosis is an important predictor of long-term function and symptoms. Long-term survivors of EOCRC experience more anxiety, and embarrassment with bowel movements, as well as negative body image when compared with their older counterparts³⁰⁸. Young patients may benefit from fertility/family planning input, employment/financial counselling, and psychosocial support. Limited

focus on functional outcomes among this patient group has led to little data on the subject. Future studies evaluating patient-reported outcome measures would identify the unmet needs of these patients, and guide the development of holistic models of care. In particular, discrete choice experimental data would help facilitate doctor-patient discussion and guide therapeutic decision making.

Collaborative platform

Collaboration represents an integral component of surgical research. Technological advances in communication and dissemination of information on a global scale, have facilitated an explosion of possibilities in collaborative research. In tandem with a paradigm shift in attitude, a united approach represents the future of surgical science. Through aggregation and analysis of large scale clinical and biological data, pathological patterns and predictive models can be derived so that surgical care can transition from a procedure-based specialty to disease-based. The data presented in this thesis represents a platform from which multiple clinical and biological studies on EOCRC can stem from. The REACCT Collaborative has united a group of surgeons/physicians with a common goal from all over the world. The analyses to date the clinical, pathological and molecular features of EOCRC, as well as oncological outcomes. Several new REACCT Collaborative studies patient-reported outcome measures, discrete choice experimental data, and evaluation of the genome and microbiome are on the horizon.

Early age onset cancer

For reasons not yet known, early age onset cancer is rising. This increase is not limited to CRC, and has also been observed in other solid organ malignancies. Questions surrounding aetiology, biomolecular profile, treatment sensitivity, and prognosis remain unanswered. Optimum preventative, diagnostic and therapeutic strategies are undefined. This patient group is largely underrepresented in clinical trials and present unique challenges. Functional status, psychosocial well-being, and quality of life in survivorship are extremely important. Expertise in early age cancer, on a multidisciplinary level (including the disciplines of surgery, oncology, genetics, nursing) is required, potentially with the emergence of early age onset cancer has a centralised subspecialty in its own right, with dedicated clinics and teams of professionals to care for these patients. Particular focus should be on appropriate multimodal treatment (with avoidance of overtreatment), consideration of age-specific concerns and long-term treatment-related morbidities, and identification of genetic mutations which may influence therapeutic decision making.

Integrating intra-tumoral immune response in the management of CRC

Individual patient factors, such as intra-tumoral immune response, may also influence oncological outcomes. Lymphocyte (T cell) infiltration is an important predictor of outcome in CRC, superior to the TNM staging system and MSI status, and varies considerably as

demonstrated in the study described in Chapter 7. Importantly, not all MSS tumours are immunologically ‘cold’. Previous studies have shown that approximately 20% will have high Immunoscores indicating robust T cell infiltration. Intra-tumoral immune response could be assessed from diagnostic biopsies pre-treatment, and could be used as an adjunct to the TNM staging system and MSI status, to categorise patients. Integration of the immune response as measured by the Immunoscore has been proposed to risk stratify patients, and guide prognosis and management, e.g. the benefit of adjuvant chemotherapy in colon cancer or suitability for immunotherapy¹⁸¹. The immune microenvironment however, is far more complex than a binary *high/hot*’ and ‘*low/cold*’ classification system based on density of T cell infiltration. Heterogeneous immune signatures characterized by checkpoint expression and pro- and anti-tumour mediators exist. These markers could be incorporated into routine histopathological analysis of diagnostic biopsies.

Intra-tumoral immune response to predict efficacy of immunotherapy

Understanding the mechanisms that result in failure to immunotherapy with checkpoint blockade and to identify immunological biomarkers that predict response to treatment are two of the most important aims of cancer immunology. Immunotherapy with checkpoint blockade has predominantly considered in patients with MSI tumours, owing to the favorable response observed in clinical trials compared to MSS tumours. Clinical efficacy is thought to be related to the relative

increase in PD-1 expression in MSI tumours. Response varies however, among patients with the same MSI status. The study described in Chapter 7 demonstrated that significant upregulation of PD-1 also occurs in a subset of MSS tumours, whilst not all MSI tumours exhibit high levels, possibly explaining the varied clinical response observed in trials. The clinical implications of this immunological heterogeneity in patients with same MSI status are several-fold. Firstly, it highlights the limitations of a binary classification system of MSI and MSS in selecting patients suitable for immunotherapy, and the need for more comprehensive categorization of tumour biology. Secondly, it broadens the spectrum of patients who may potentially benefit from immunotherapy as there may be a subset with MSS disease who may respond. Likewise, there are a subset of patients with MSI tumours who will not respond well to immunotherapy. Tumour (mutational burden, heterogeneity) and immune (checkpoint expression) biomarkers predictive of response need to be identified, and their presence evaluated in diagnostic biopsies or surgical specimens using reliable, reproducible, cost-effective techniques. As flow cytometric analysis and single cell RNA sequencing are not feasible on a large scale in clinical practice, immunohistochemistry staining of specific markers may represent a more realistic option. In addition, validated scoring systems are required to meaningfully interpret and translate the results into clinical practice.

9.2 Conclusion

In conclusion, microsatellite instability in patients with early age onset colorectal cancer is associated with distinct immunopathological patterns in comparison to those with later onset disease. Inhibitory checkpoint expression varies significantly among patients with the same microsatellite status, suggesting microsatellite status in isolation may not accurately predict response to immunotherapy. Colorectal tumours contain pro-tumour immune cell phenotypes such as amphiregulin-producing $\gamma\delta$ T cells which may represent potential immunotherapeutic targets. Together, these studies increase our understanding of the immunopathological features of microsatellite instability in early age onset colorectal cancer, and the immune landscape of all age colorectal cancer. The work presented translates from bedside to bench and back again, supporting the immune response as a viable therapeutic target in colorectal cancer.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* 01 2018;68(1):7-30. doi:10.3322/caac.21442
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 11 2018;68(6):394-424. doi:10.3322/caac.21492
3. Islami F, Goding Sauer A, Miller KD, et al. Proportion and number of cancer cases and deaths attributable to potentially modifiable risk factors in the United States. *CA Cancer J Clin.* 01 2018;68(1):31-54. doi:10.3322/caac.21440
4. Siegel RL, Miller KD, Goding Sauer A, et al. Colorectal cancer statistics, 2020. *CA Cancer J Clin.* 05 2020;70(3):145-164. doi:10.3322/caac.21601
5. Carethers JM, Doubeni CA. Causes of Socioeconomic Disparities in Colorectal Cancer and Intervention Framework and Strategies. *Gastroenterology.* 01 2020;158(2):354-367. doi:10.1053/j.gastro.2019.10.029
6. Doubeni CA, Laiyemo AO, Major JM, et al. Socioeconomic status and the risk of colorectal cancer: an analysis of more than a half million adults in the National Institutes of Health-AARP Diet and Health Study. *Cancer.* Jul 2012;118(14):3636-44. doi:10.1002/cncr.26677
7. Brenner H, Hoffmeister M, Arndt V, Haug U. Gender differences in colorectal cancer: implications for age at initiation of screening. *Br J Cancer.* Mar 2007;96(5):828-31. doi:10.1038/sj.bjc.6603628
8. Vuik FE, Nieuwenburg SA, Bardou M, et al. Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years. *Gut.* May 2019;doi:10.1136/gutjnl-2018-317592
9. Bailey CE, Hu CY, You YN, et al. Increasing disparities in the age-related incidences of colon and rectal cancers in the United States, 1975-2010. *JAMA Surg.* Jan 2015;150(1):17-22. doi:10.1001/jamasurg.2014.1756
10. White A, Ironmonger L, Steele RJC, Ormiston-Smith N, Crawford C, Seims A. A review of sex-related differences in colorectal cancer incidence, screening uptake, routes to diagnosis, cancer stage and survival in the UK. *BMC Cancer.* Sep 2018;18(1):906. doi:10.1186/s12885-018-4786-7
11. Mehta RS, Song M, Nishihara R, et al. Dietary Patterns and Risk of Colorectal Cancer: Analysis by Tumor Location and Molecular Subtypes. *Gastroenterology.* 06 2017;152(8):1944-1953.e1. doi:10.1053/j.gastro.2017.02.015
12. Liang PS, Chen TY, Giovannucci E. Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. *Int J Cancer.* May 2009;124(10):2406-15. doi:10.1002/ijc.24191
13. Bardou M, Barkun AN, Martel M. Obesity and colorectal cancer. *Gut.* Jun 2013;62(6):933-47. doi:10.1136/gutjnl-2013-304701
14. Fedirko V, Tramacere I, Bagnardi V, et al. Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis

- of published studies. *Ann Oncol.* Sep 2011;22(9):1958-1972. doi:10.1093/annonc/mdq653
15. Wong SH, Yu J. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. *Nat Rev Gastroenterol Hepatol.* 11 2019;16(11):690-704. doi:10.1038/s41575-019-0209-8
 16. Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med.* Jul 2000;343(2):78-85. doi:10.1056/NEJM200007133430201
 17. Houlston RS, Cheadle J, Dobbins SE, et al. Meta-analysis of three genome-wide association studies identifies susceptibility loci for colorectal cancer at 1q41, 3q26.2, 12q13.13 and 20q13.33. *Nat Genet.* Nov 2010;42(11):973-7. doi:10.1038/ng.670
 18. Peters U, Jiao S, Schumacher FR, et al. Identification of Genetic Susceptibility Loci for Colorectal Tumors in a Genome-Wide Meta-analysis. *Gastroenterology.* Apr 2013;144(4):799-807.e24. doi:10.1053/j.gastro.2012.12.020
 19. Tomlinson IP, Webb E, Carvajal-Carmona L, et al. A genome-wide association study identifies colorectal cancer susceptibility loci on chromosomes 10p14 and 8q23.3. *Nat Genet.* May 2008;40(5):623-30. doi:10.1038/ng.111
 20. Syngal S, Brand RE, Church JM, et al. ACG clinical guideline: Genetic testing and management of hereditary gastrointestinal cancer syndromes. *Am J Gastroenterol.* Feb 2015;110(2):223-62; quiz 263. doi:10.1038/ajg.2014.435
 21. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* Jun 1990;61(5):759-67. doi:10.1016/0092-8674(90)90186-i
 22. Kolligs FT, Bommer G, Göke B. Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion.* 2002;66(3):131-44. doi:10.1159/000066755
 23. Bos JL, Fearon ER, Hamilton SR, et al. Prevalence of ras gene mutations in human colorectal cancers. *Nature.* 1987 May 28-Jun 3 1987;327(6120):293-7. doi:10.1038/327293a0
 24. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature.* 1987 May 28-Jun 3 1987;327(6120):298-303. doi:10.1038/327298a0
 25. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology.* Jun 2010;138(6):2059-72. doi:10.1053/j.gastro.2009.12.065
 26. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med.* Mar 2003;348(10):919-32. doi:10.1056/NEJMra012242
 27. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut.* Jul 2006;55(7):1000-6. doi:10.1136/gut.2005.082933
 28. Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology.* May 2002;122(5):1376-87. doi:10.1053/gast.2002.32997

29. Cheng YW, Pincas H, Bacolod MD, et al. CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. *Clin Cancer Res*. Oct 2008;14(19):6005-13. doi:10.1158/1078-0432.CCR-08-0216
30. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med*. Nov 2015;21(11):1350-6. doi:10.1038/nm.3967
31. Isella C, Brundu F, Bellomo SE, et al. Selective analysis of cancer-cell intrinsic transcriptional traits defines novel clinically relevant subtypes of colorectal cancer. *Nat Commun*. 05 2017;8:15107. doi:10.1038/ncomms15107
32. Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. *Gastroenterology*. Jan 2015;148(1):77-87.e2. doi:10.1053/j.gastro.2014.09.038
33. Hohenberger W, Weber K, Matzel K, Papadopoulos T, Merkel S. Standardized surgery for colonic cancer: complete mesocolic excision and central ligation--technical notes and outcome. *Colorectal Dis*. May 2009;11(4):354-64; discussion 364-5. doi:10.1111/j.1463-1318.2008.01735.x
34. Heald RJ, Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. *Lancet*. Jun 1986;1(8496):1479-82. doi:10.1016/s0140-6736(86)91510-2
35. Bach SP, Hill J, Monson JR, et al. A predictive model for local recurrence after transanal endoscopic microsurgery for rectal cancer. *Br J Surg*. Mar 2009;96(3):280-90. doi:10.1002/bjs.6456
36. Fujita S, Mizusawa J, Kanemitsu Y, et al. Mesorectal Excision With or Without Lateral Lymph Node Dissection for Clinical Stage II/III Lower Rectal Cancer (JCOG0212): A Multicenter, Randomized Controlled, Noninferiority Trial. *Ann Surg*. 08 2017;266(2):201-207. doi:10.1097/SLA.0000000000002212
37. Shida D, Kanemitsu Y, Hamaguchi T, Shimada Y. Introducing the eighth edition of the tumor-node-metastasis classification as relevant to colorectal cancer, anal cancer and appendiceal cancer: a comparison study with the seventh edition of the tumor-node-metastasis and the Japanese Classification of Colorectal, Appendiceal, and Anal Carcinoma. *Jpn J Clin Oncol*. Apr 2019;49(4):321-328. doi:10.1093/jjco/hyy198
38. Williamson JS, Quyn AJ, Sagar PM. Rectal cancer lateral pelvic sidewall lymph nodes: a review of controversies and management. *Br J Surg*. Nov 2020;107(12):1562-1569. doi:10.1002/bjs.11925
39. Quyn AJ, Austin KK, Young JM, et al. Outcomes of pelvic exenteration for locally advanced primary rectal cancer: Overall survival and quality of life. *Eur J Surg Oncol*. Jun 2016;42(6):823-8. doi:10.1016/j.ejso.2016.02.016
40. Solum AM, Riffenburgh RH, Johnstone PA. Survival of patients with untreated rectal cancer. *J Surg Oncol*. Sep 2004;87(4):157-61. doi:10.1002/jso.20104
41. Collaborative P. Surgical and Survival Outcomes Following Pelvic Exenteration for Locally Advanced Primary Rectal Cancer:

- Results From an International Collaboration. *Ann Surg.* 02 2019;269(2):315-321. doi:10.1097/SLA.0000000000002528
42. Bonjer HJ, Deijen CL, Abis GA, et al. A randomized trial of laparoscopic versus open surgery for rectal cancer. *N Engl J Med.* Apr 2015;372(14):1324-32. doi:10.1056/NEJMoa1414882
 43. Jeong SY, Park JW, Nam BH, et al. Open versus laparoscopic surgery for mid-rectal or low-rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial): survival outcomes of an open-label, non-inferiority, randomised controlled trial. *Lancet Oncol.* 06 2014;15(7):767-74. doi:10.1016/S1470-2045(14)70205-0
 44. Fleshman J, Branda M, Sargent DJ, et al. Effect of Laparoscopic-Assisted Resection vs Open Resection of Stage II or III Rectal Cancer on Pathologic Outcomes: The ACOSOG Z6051 Randomized Clinical Trial. *JAMA.* Oct 2015;314(13):1346-55. doi:10.1001/jama.2015.10529
 45. Miskovic D, Ahmed J, Bissett-Amess R, et al. European consensus on the standardization of robotic total mesorectal excision for rectal cancer. *Colorectal Dis.* 03 2019;21(3):270-276. doi:10.1111/codi.14502
 46. Wasmuth HH, Faerden AE, Myklebust T, et al. Transanal total mesorectal excision for rectal cancer has been suspended in Norway. *Br J Surg.* 01 2020;107(1):121-130. doi:10.1002/bjs.11459
 47. Sauer R, Liersch T, Merkel S, et al. Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. *J Clin Oncol.* Jun 2012;30(16):1926-33. doi:10.1200/JCO.2011.40.1836
 48. Cedermark B, Dahlberg M, Glimelius B, et al. Improved survival with preoperative radiotherapy in resectable rectal cancer. *N Engl J Med.* 04 1997;336(14):980-7. doi:10.1056/NEJM199704033361402
 49. Kuebler JP, Wieand HS, O'Connell MJ, et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. *J Clin Oncol.* Jun 2007;25(16):2198-204. doi:10.1200/JCO.2006.08.2974
 50. André T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol.* Jul 2009;27(19):3109-16. doi:10.1200/JCO.2008.20.6771
 51. Sargent D, Sobrero A, Grothey A, et al. Evidence for cure by adjuvant therapy in colon cancer: observations based on individual patient data from 20,898 patients on 18 randomized trials. *J Clin Oncol.* Feb 2009;27(6):872-7. doi:10.1200/JCO.2008.19.5362
 52. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med.* Feb 1990;322(6):352-8. doi:10.1056/NEJM199002083220602
 53. Wolmark N, Rockette H, Fisher B, et al. The benefit of leucovorin-modulated fluorouracil as postoperative adjuvant therapy for primary colon cancer: results from National Surgical Adjuvant

- Breast and Bowel Project protocol C-03. *J Clin Oncol*. Oct 1993;11(10):1879-87. doi:10.1200/JCO.1993.11.10.1879
54. O'Connell MJ, Mailliard JA, Kahn MJ, et al. Controlled trial of fluorouracil and low-dose leucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. *J Clin Oncol*. Jan 1997;15(1):246-50. doi:10.1200/JCO.1997.15.1.246
 55. André T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med*. Jun 2004;350(23):2343-51. doi:10.1056/NEJMoa032709
 56. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *J Clin Oncol*. Oct 2011;29(28):3768-74. doi:10.1200/JCO.2011.36.4539
 57. Haller DG, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol*. Apr 2011;29(11):1465-71. doi:10.1200/JCO.2010.33.6297
 58. Grothey A, Sobrero AF, Shields AF, et al. Duration of Adjuvant Chemotherapy for Stage III Colon Cancer. *N Engl J Med*. Mar 2018;378(13):1177-1188. doi:10.1056/NEJMoa1713709
 59. Argilés G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 10 2020;31(10):1291-1305. doi:10.1016/j.annonc.2020.06.022
 60. Peeters KC, Marijnen CA, Nagtegaal ID, et al. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg*. Nov 2007;246(5):693-701. doi:10.1097/01.sla.0000257358.56863.ce
 61. Bosset JF, Collette L, Calais G, et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med*. Sep 2006;355(11):1114-23. doi:10.1056/NEJMoa060829
 62. Martin ST, Heneghan HM, Winter DC. Systematic review and meta-analysis of outcomes following pathological complete response to neoadjuvant chemoradiotherapy for rectal cancer. *Br J Surg*. Jul 2012;99(7):918-28. doi:10.1002/bjs.8702
 63. Glynne-Jones R, Counsell N, Quirke P, et al. Chronicle: results of a randomised phase III trial in locally advanced rectal cancer after neoadjuvant chemoradiation randomising postoperative adjuvant capecitabine plus oxaliplatin (XELOX) versus control. *Ann Oncol*. Jul 2014;25(7):1356-62. doi:10.1093/annonc/mdu147
 64. Sainato A, Cernusco Luna Nunzia V, Valentini V, et al. No benefit of adjuvant Fluorouracil Leucovorin chemotherapy after neoadjuvant chemoradiotherapy in locally advanced cancer of the rectum (LARC): Long term results of a randomized trial (I-CNR-RT). *Radiother Oncol*. Nov 2014;113(2):223-9. doi:10.1016/j.radonc.2014.10.006
 65. Breugom AJ, van Gijn W, Muller EW, et al. Adjuvant chemotherapy for rectal cancer patients treated with preoperative (chemo)radiotherapy and total mesorectal excision: a Dutch Colorectal Cancer Group (DCCG) randomized phase III trial. *Ann Oncol*. Apr 2015;26(4):696-701. doi:10.1093/annonc/mdu560

66. Bosset JF, Calais G, Mineur L, et al. Fluorouracil-based adjuvant chemotherapy after preoperative chemoradiotherapy in rectal cancer: long-term results of the EORTC 22921 randomised study. *Lancet Oncol.* Feb 2014;15(2):184-90. doi:10.1016/S1470-2045(13)70599-0
67. Zaborowski A, Stakelum A, Winter DC. Systematic review of outcomes after total neoadjuvant therapy for locally advanced rectal cancer. *Br J Surg.* Jul 2019;106(8):979-987. doi:10.1002/bjs.11171
68. Petrelli F, Trevisan F, Cabiddu M, et al. Total Neoadjuvant Therapy in Rectal Cancer: A Systematic Review and Meta-analysis of Treatment Outcomes. *Ann Surg.* Jul 2019;doi:10.1097/SLA.0000000000003471
69. Ryan OK, Ryan É, Creavin B, Boland MR, Kelly ME, Winter DC. Systematic review and meta-analysis comparing primary resection and anastomosis versus Hartmann's procedure for the management of acute perforated diverticulitis with generalised peritonitis. *Tech Coloproctol.* Mar 2020;doi:10.1007/s10151-020-02172-2
70. Kalady MF, de Campos-Lobato LF, Stocchi L, et al. Predictive factors of pathologic complete response after neoadjuvant chemoradiation for rectal cancer. *Ann Surg.* Oct 2009;250(4):582-9. doi:10.1097/SLA.0b013e3181b91e63
71. Tulchinsky H, Shmueli E, Figer A, Klausner JM, Rabau M. An interval >7 weeks between neoadjuvant therapy and surgery improves pathologic complete response and disease-free survival in patients with locally advanced rectal cancer. *Ann Surg Oncol.* Oct 2008;15(10):2661-7. doi:10.1245/s10434-008-9892-3
72. Lefevre JH, Mineur L, Kotti S, et al. Effect of Interval (7 or 11 weeks) Between Neoadjuvant Radiochemotherapy and Surgery on Complete Pathologic Response in Rectal Cancer: A Multicenter, Randomized, Controlled Trial (GRECCAR-6). *J Clin Oncol.* 11 2016;34(31):3773-3780. doi:10.1200/JCO.2016.67.6049
73. Bryant CL, Lunniss PJ, Knowles CH, Thaha MA, Chan CL. Anterior resection syndrome. *Lancet Oncol.* Sep 2012;13(9):e403-8. doi:10.1016/S1470-2045(12)70236-X
74. Pieniowski EHA, Palmer GJ, Juul T, et al. Low Anterior Resection Syndrome and Quality of Life After Sphincter-Sparing Rectal Cancer Surgery: A Long-term Longitudinal Follow-up. *Dis Colon Rectum.* 01 2019;62(1):14-20. doi:10.1097/DCR.0000000000001228
75. van Heinsbergen M, Janssen-Heijnen ML, Leijtens JW, Slooter GD, Konsten JL. Bowel dysfunction after sigmoid resection underestimated: Multicentre study on quality of life after surgery for carcinoma of the rectum and sigmoid. *Eur J Surg Oncol.* 08 2018;44(8):1261-1267. doi:10.1016/j.ejso.2018.05.003
76. Benatti P, Gafà R, Barana D, et al. Microsatellite instability and colorectal cancer prognosis. *Clin Cancer Res.* Dec 2005;11(23):8332-40. doi:10.1158/1078-0432.CCR-05-1030
77. Des Guetz G, Schischmanoff O, Nicolas P, Perret GY, Morere JF, Uzzan B. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review

- with meta-analysis. *Eur J Cancer*. Jul 2009;45(10):1890-6. doi:10.1016/j.ejca.2009.04.018
78. Martin LM, Marples B, Coffey M, et al. DNA mismatch repair and the DNA damage response to ionizing radiation: making sense of apparently conflicting data. *Cancer Treat Rev*. Nov 2010;36(7):518-27. doi:10.1016/j.ctrv.2010.03.008
79. Yan T, Seo Y, Kinsella TJ. Differential cellular responses to prolonged LDR-IR in MLH1-proficient and MLH1-deficient colorectal cancer HCT116 cells. *Clin Cancer Res*. Nov 2009;15(22):6912-20. doi:10.1158/1078-0432.CCR-09-1698
80. Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K, Morimatsu M. An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. *Cancer*. Feb 1989;63(3):539-43. doi:10.1002/1097-0142(19890201)63:3<539::aid-cncr2820630323>3.0.co;2-s
81. Hase K, Shatney C, Johnson D, Trollope M, Vierra M. Prognostic value of tumor "budding" in patients with colorectal cancer. *Dis Colon Rectum*. Jul 1993;36(7):627-35. doi:10.1007/BF02238588
82. Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology*. Feb 2002;40(2):127-32. doi:10.1046/j.1365-2559.2002.01324.x
83. Kevans D, Wang LM, Sheahan K, et al. Epithelial-mesenchymal transition (EMT) protein expression in a cohort of stage II colorectal cancer patients with characterized tumor budding and mismatch repair protein status. *Int J Surg Pathol*. Dec 2011;19(6):751-60. doi:10.1177/1066896911414566
84. Rogers AC, Winter DC, Heeney A, et al. Systematic review and meta-analysis of the impact of tumour budding in colorectal cancer. *Br J Cancer*. 09 2016;115(7):831-40. doi:10.1038/bjc.2016.274
85. Rogers AC, Gibbons D, Hanly AM, et al. Prognostic significance of tumor budding in rectal cancer biopsies before neoadjuvant therapy. *Mod Pathol*. Jan 2014;27(1):156-62. doi:10.1038/modpathol.2013.124
86. Jäger T, Neureiter D, Fallaha M, et al. The potential predictive value of tumor budding for neoadjuvant chemoradiotherapy response in locally advanced rectal cancer. *Strahlenther Onkol*. Nov 2018;194(11):991-1006. doi:10.1007/s00066-018-1340-0
87. Demir A, Alan O, Oruc E. Tumor budding for predicting prognosis of resected rectum cancer after neoadjuvant treatment. *World J Surg Oncol*. Mar 2019;17(1):50. doi:10.1186/s12957-019-1588-6
88. Du C, Xue W, Li J, Cai Y, Gu J. Morphology and prognostic value of tumor budding in rectal cancer after neoadjuvant radiotherapy. *Hum Pathol*. Jul 2012;43(7):1061-7. doi:10.1016/j.humpath.2011.07.026
89. Lugli A, Karamitopoulou E, Panayiotides I, et al. CD8+ lymphocytes/ tumour-budding index: an independent prognostic factor representing a 'pro-/anti-tumour' approach to tumour host interaction

- in colorectal cancer. *Br J Cancer*. Oct 2009;101(8):1382-92. doi:10.1038/sj.bjc.6605318
90. Symonds DA, Vickery AL. Mucinous carcinoma of the colon and rectum. *Cancer*. Apr 1976;37(4):1891-1900. doi:10.1002/1097-0142(197604)37:4<1891::aid-cnrcr2820370439>3.0.co;2-z
 91. Kang H, O'Connell JB, Maggard MA, Sack J, Ko CY. A 10-year outcomes evaluation of mucinous and signet-ring cell carcinoma of the colon and rectum. *Dis Colon Rectum*. Jun 2005;48(6):1161-8. doi:10.1007/s10350-004-0932-1
 92. Simha V, Kapoor R, Gupta R, Bahl A, Nada R. Mucinous adenocarcinoma of the rectum: a poor candidate for neo-adjuvant chemoradiation? *J Gastrointest Oncol*. Aug 2014;5(4):276-9. doi:10.3978/j.issn.2078-6891.2014.020
 93. McCawley N, Clancy C, O'Neill BD, Deasy J, McNamara DA, Burke JP. Mucinous Rectal Adenocarcinoma Is Associated with a Poor Response to Neoadjuvant Chemoradiotherapy: A Systematic Review and Meta-analysis. *Dis Colon Rectum*. Dec 2016;59(12):1200-1208. doi:10.1097/DCR.0000000000000635
 94. Shin US, Yu CS, Kim JH, et al. Mucinous rectal cancer: effectiveness of preoperative chemoradiotherapy and prognosis. *Ann Surg Oncol*. Aug 2011;18(8):2232-9. doi:10.1245/s10434-011-1612-8
 95. Hugen N, Verhoeven RH, Radema SA, et al. Prognosis and value of adjuvant chemotherapy in stage III mucinous colorectal carcinoma. *Ann Oncol*. Nov 2013;24(11):2819-24. doi:10.1093/annonc/mdt378
 96. Negri FV, Wotherspoon A, Cunningham D, Norman AR, Chong G, Ross PJ. Mucinous histology predicts for reduced fluorouracil responsiveness and survival in advanced colorectal cancer. *Ann Oncol*. Aug 2005;16(8):1305-10. doi:10.1093/annonc/mdi244
 97. Reynolds IS, Furney SJ, Kay EW, McNamara DA, Prehn JHM, Burke JP. Meta-analysis of the molecular associations of mucinous colorectal cancer. *Br J Surg*. 05 2019;106(6):682-691. doi:10.1002/bjs.11142
 98. Kong JC, Guerra GR, Warriar SK, et al. Prognostic value of tumour regression grade in locally advanced rectal cancer: a systematic review and meta-analysis. *Colorectal Dis*. 07 2018;20(7):574-585. doi:10.1111/codi.14106
 99. Jacobs D, Zhu R, Luo J, et al. Defining Early-Onset Colon and Rectal Cancers. *Front Oncol*. 2018;8:504. doi:10.3389/fonc.2018.00504
 100. Chambers AC, Dixon SW, White P, Williams AC, Thomas MG, Messenger DE. Demographic trends in the incidence of young-onset colorectal cancer: a population-based study. *Br J Surg*. Apr 2020;107(5):595-605. doi:10.1002/bjs.11486
 101. Lui RN, Tsoi KKF, Ho JMW, et al. Global Increasing Incidence of Young-Onset Colorectal Cancer Across 5 Continents: A Joinpoint Regression Analysis of 1,922,167 Cases. *Cancer Epidemiol Biomarkers Prev*. 08 2019;28(8):1275-1282. doi:10.1158/1055-9965.EPI-18-1111
 102. Bhandari A, Woodhouse M, Gupta S. Colorectal cancer is a leading cause of cancer incidence and mortality among adults younger

- than 50 years in the USA: a SEER-based analysis with comparison to other young-onset cancers. *J Investig Med*. 02 2017;65(2):311-315. doi:10.1136/jim-2016-000229
103. Willauer AN, Liu Y, Pereira AAL, et al. Clinical and molecular characterization of early-onset colorectal cancer. *Cancer*. 06 2019;125(12):2002-2010. doi:10.1002/cncr.31994
 104. Meyer JE, Narang T, Schnoll-Sussman FH, Pochapin MB, Christos PJ, Sherr DL. Increasing incidence of rectal cancer in patients aged younger than 40 years: an analysis of the surveillance, epidemiology, and end results database. *Cancer*. Sep 2010;116(18):4354-9. doi:10.1002/cncr.25432
 105. Siegel RL, Jemal A, Ward EM. Increase in incidence of colorectal cancer among young men and women in the United States. *Cancer Epidemiol Biomarkers Prev*. Jun 2009;18(6):1695-8. doi:10.1158/1055-9965.EPI-09-0186
 106. Saltzstein SL, Behling CA. Age and time as factors in the left-to-right shift of the subsite of colorectal adenocarcinoma: a study of 213,383 cases from the California Cancer Registry. *J Clin Gastroenterol*. Feb 2007;41(2):173-7. doi:10.1097/01.mcg.0000225550.26751.6a
 107. Suttie SA, Shaikh I, Mullen R, Amin AI, Daniel T, Yalamarthi S. Outcome of right- and left-sided colonic and rectal cancer following surgical resection. *Colorectal Dis*. Aug 2011;13(8):884-9. doi:10.1111/j.1463-1318.2010.02356.x
 108. Lim DR, Kuk JK, Kim T, Shin EJ. Comparison of oncological outcomes of right-sided colon cancer versus left-sided colon cancer after curative resection: Which side is better outcome? *Medicine (Baltimore)*. Oct 2017;96(42):e8241. doi:10.1097/MD.00000000000008241
 109. Powell AG, Wallace R, McKee RF, et al. The relationship between tumour site, clinicopathological characteristics and cancer-specific survival in patients undergoing surgery for colorectal cancer. *Colorectal Dis*. Dec 2012;14(12):1493-9. doi:10.1111/j.1463-1318.2012.03048.x
 110. Liang JT, Huang KC, Cheng AL, Jeng YM, Wu MS, Wang SM. Clinicopathological and molecular biological features of colorectal cancer in patients less than 40 years of age. *Br J Surg*. Feb 2003;90(2):205-14. doi:10.1002/bjs.4015
 111. You YN, Xing Y, Feig BW, Chang GJ, Cormier JN. Young-onset colorectal cancer: is it time to pay attention? *Arch Intern Med*. Feb 2012;172(3):287-9. doi:10.1001/archinternmed.2011.602
 112. O'Connell JB, Maggard MA, Liu JH, Etzioni DA, Livingston EH, Ko CY. Do young colon cancer patients have worse outcomes? *World J Surg*. Jun 2004;28(6):558-62.
 113. Saraste D, Järås J, Martling A. Population-based analysis of outcomes with early-age colorectal cancer. *Br J Surg*. Feb 2020;107(3):301-309. doi:10.1002/bjs.11333
 114. Kneuertz PJ, Chang GJ, Hu CY, et al. Overtreatment of young adults with colon cancer: more intense treatments with unmatched survival gains. *JAMA Surg*. May 2015;150(5):402-9. doi:10.1001/jamasurg.2014.3572

115. O'Connell JB, Maggard MA, Livingston EH, Yo CK. Colorectal cancer in the young. *Am J Surg*. Mar 2004;187(3):343-8. doi:10.1016/j.amjsurg.2003.12.020
116. Dozois EJ, Boardman LA, Suwanthanma W, et al. Young-onset colorectal cancer in patients with no known genetic predisposition: can we increase early recognition and improve outcome? *Medicine (Baltimore)*. Sep 2008;87(5):259-63. doi:10.1097/MD.0b013e3181881354
117. Connell LC, Mota JM, Braghiroli MI, Hoff PM. The Rising Incidence of Younger Patients With Colorectal Cancer: Questions About Screening, Biology, and Treatment. *Curr Treat Options Oncol*. 04 2017;18(4):23. doi:10.1007/s11864-017-0463-3
118. Chang DT, Pai RK, Rybicki LA, et al. Clinicopathologic and molecular features of sporadic early-onset colorectal adenocarcinoma: an adenocarcinoma with frequent signet ring cell differentiation, rectal and sigmoid involvement, and adverse morphologic features. *Mod Pathol*. Aug 2012;25(8):1128-39. doi:10.1038/modpathol.2012.61
119. Antelo M, Balaguer F, Shia J, et al. A high degree of LINE-1 hypomethylation is a unique feature of early-onset colorectal cancer. *PLoS One*. 2012;7(9):e45357. doi:10.1371/journal.pone.0045357
120. Lieu CH, Golemis EA, Serebriiskii IG, et al. Comprehensive Genomic Landscapes in Early and Later Onset Colorectal Cancer. *Clin Cancer Res*. 10 2019;25(19):5852-5858. doi:10.1158/1078-0432.CCR-19-0899
121. Serebriiskii IG, Connelly C, Frampton G, et al. Comprehensive characterization of RAS mutations in colon and rectal cancers in old and young patients. *Nat Commun*. 08 2019;10(1):3722. doi:10.1038/s41467-019-11530-0
122. Eriksson J, Amonkar M, Al-Jassar G, et al. Mismatch Repair/Microsatellite Instability Testing Practices among US Physicians Treating Patients with Advanced/Metastatic Colorectal Cancer. *J Clin Med*. Apr 2019;8(4)doi:10.3390/jcm8040558
123. Seppälä TT, Latchford A, Negoi I, et al. European guidelines from the EHTG and ESCP for Lynch syndrome: an updated third edition of the Mallorca guidelines based on gene and gender. *Br J Surg*. Sep 2020;doi:10.1002/bjs.11902
124. Kirzin S, Marisa L, Guimbaud R, et al. Sporadic early-onset colorectal cancer is a specific sub-type of cancer: a morphological, molecular and genetics study. *PLoS One*. 2014;9(8):e103159. doi:10.1371/journal.pone.0103159
125. Arriba M, García JL, Rueda D, et al. Unsupervised Analysis of Array Comparative Genomic Hybridization Data from Early-Onset Colorectal Cancer Reveals Equivalence with Molecular Classification and Phenotypes. *Neoplasia*. 01 2017;19(1):28-34. doi:10.1016/j.neo.2016.11.006
126. Hofseth LJ, Hebert JR, Chanda A, et al. Early-onset colorectal cancer: initial clues and current views. *Nat Rev Gastroenterol Hepatol*. 06 2020;17(6):352-364. doi:10.1038/s41575-019-0253-4
127. Liu PH, Wu K, Ng K, et al. Association of Obesity With Risk of Early-Onset Colorectal Cancer Among Women. *JAMA Oncol*. 01 2019;5(1):37-44. doi:10.1001/jamaoncol.2018.4280

128. Hidayat K, Yang CM, Shi BM. Body fatness at an early age and risk of colorectal cancer. *Int J Cancer*. 02 2018;142(4):729-740. doi:10.1002/ijc.31100
129. Goel A, Nagasaka T, Spiegel J, Meyer R, Lichliter WE, Boland CR. Low frequency of Lynch syndrome among young patients with non-familial colorectal cancer. *Clin Gastroenterol Hepatol*. Nov 2010;8(11):966-71. doi:10.1016/j.cgh.2010.06.030
130. Pearlman R, Frankel WL, Swanson B, et al. Prevalence and Spectrum of Germline Cancer Susceptibility Gene Mutations Among Patients With Early-Onset Colorectal Cancer. *JAMA Oncol*. Apr 2017;3(4):464-471. doi:10.1001/jamaoncol.2016.5194
131. Mork ME, You YN, Ying J, et al. High Prevalence of Hereditary Cancer Syndromes in Adolescents and Young Adults With Colorectal Cancer. *J Clin Oncol*. Nov 2015;33(31):3544-9. doi:10.1200/JCO.2015.61.4503
132. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-2015. *Nat Rev Cancer*. 03 2015;15(3):181-94. doi:10.1038/nrc3878
133. Sehgal R, Sheahan K, O'Connell PR, Hanly AM, Martin ST, Winter DC. Lynch syndrome: an updated review. *Genes (Basel)*. Jun 2014;5(3):497-507. doi:10.3390/genes5030497
134. Stoffel EM, Koeppe E, Everett J, et al. Germline Genetic Features of Young Individuals With Colorectal Cancer. *Gastroenterology*. 03 2018;154(4):897-905.e1. doi:10.1053/j.gastro.2017.11.004
135. Zaborowski AM, Murphy B, Creavin B, et al. Clinicopathological features and oncological outcomes of patients with young-onset rectal cancer. *Br J Surg*. Apr 2020;107(5):606-612. doi:10.1002/bjs.11526
136. Kolarich A, George TJ, Hughes SJ, et al. Rectal cancer patients younger than 50 years lack a survival benefit from NCCN guideline-directed treatment for stage II and III disease. *Cancer*. Sep 2018;124(17):3510-3519. doi:10.1002/cncr.31527
137. Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med*. Jun 2015;372(26):2509-20. doi:10.1056/NEJMoa1500596
138. Montecino-Rodriguez E, Berent-Maoz B, Dorshkind K. Causes, consequences, and reversal of immune system aging. *J Clin Invest*. Mar 2013;123(3):958-65. doi:10.1172/JCI64096
139. BURNET M. Cancer; a biological approach. I. The processes of control. *Br Med J*. Apr 1957;1(5022):779-86. doi:10.1136/bmj.1.5022.779
140. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. Mar 2011;331(6024):1565-70. doi:10.1126/science.1203486
141. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. Mar 2011;144(5):646-74. doi:10.1016/j.cell.2011.02.013
142. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol*. Apr 2014;27:16-25. doi:10.1016/j.coi.2014.01.004

143. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. Sep 2006;313(5795):1960-4. doi:10.1126/science.1129139
144. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov*. Mar 2019;18(3):197-218. doi:10.1038/s41573-018-0007-y
145. Pagès F, Mlecnik B, Marliot F, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet*. 05 2018;391(10135):2128-2139. doi:10.1016/S0140-6736(18)30789-X
146. Berg RE, Forman J. The role of CD8 T cells in innate immunity and in antigen non-specific protection. *Curr Opin Immunol*. Jun 2006;18(3):338-43. doi:10.1016/j.coi.2006.03.010
147. Raskov H, Orhan A, Christensen JP, Gögenur I. Cytotoxic CD8. *Br J Cancer*. 01 2021;124(2):359-367. doi:10.1038/s41416-020-01048-4
148. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 03 15 2012;12(4):298-306. doi:10.1038/nrc3245
149. Broussard EK, Disis ML. TNM staging in colorectal cancer: T is for T cell and M is for memory. *J Clin Oncol*. Feb 20 2011;29(6):601-3. doi:10.1200/JCO.2010.32.9078
150. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene*. Feb 25 2010;29(8):1093-102. doi:10.1038/onc.2009.416
151. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4⁺T cells: differentiation and functions. *Clin Dev Immunol*. 2012;2012:925135. doi:10.1155/2012/925135
152. Corthay A. How do regulatory T cells work? *Scand J Immunol*. Oct 2009;70(4):326-36. doi:10.1111/j.1365-3083.2009.02308.x
153. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol*. Apr 2006;6(4):295-307. doi:10.1038/nri1806
154. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. Jul 2008;8(7):523-32. doi:10.1038/nri2343
155. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol*. May 2008;9(5):503-10. doi:10.1038/ni1582
156. Paul S, Lal G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Front Immunol*. 2017;8:1124. doi:10.3389/fimmu.2017.01124
157. Kumar V, McNerney ME. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nat Rev Immunol*. May 2005;5(5):363-74. doi:10.1038/nri1603
158. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol Rev*. Dec 2006;214:143-54. doi:10.1111/j.1600-065X.2006.00458.x

159. O'Neill C, Cassidy FC, O'Shea D, Hogan AE. Mucosal Associated Invariant T Cells in Cancer-Friend or Foe? *Cancers (Basel)*. Mar 30 2021;13(7)doi:10.3390/cancers13071582
160. Dusseaux M, Martin E, Serriari N, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood*. Jan 27 2011;117(4):1250-9. doi:10.1182/blood-2010-08-303339
161. Hinks TSC, Zhang XW. MAIT Cell Activation and Functions. *Front Immunol*. 2020;11:1014. doi:10.3389/fimmu.2020.01014
162. Pisarska MM, Dunne MR, O'Shea D, Hogan AE. Interleukin-17 producing mucosal associated invariant T cells - emerging players in chronic inflammatory diseases? *Eur J Immunol*. 08 2020;50(8):1098-1108. doi:10.1002/eji.202048645
163. Lamichhane R, Schneider M, de la Harpe SM, et al. TCR- or Cytokine-Activated CD8. *Cell Rep*. Sep 17 2019;28(12):3061-3076.e5. doi:10.1016/j.celrep.2019.08.054
164. Le Bourhis L, Martin E, Péguillet I, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol*. Aug 2010;11(8):701-8. doi:10.1038/ni.1890
165. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer immunotherapy with $\gamma\delta$ T cells: many paths ahead of us. *Cell Mol Immunol*. 09 2020;17(9):925-939. doi:10.1038/s41423-020-0504-x
166. Rampoldi F, Prinz I. Three Layers of Intestinal $\gamma\delta$ T Cells Talk Different Languages With the Microbiota. *Front Immunol*. 2022;13:849954. doi:10.3389/fimmu.2022.849954
167. Zhao Y, Niu C, Cui J. Gamma-delta ($\gamma\delta$) T cells: friend or foe in cancer development? *J Transl Med*. 01 10 2018;16(1):3. doi:10.1186/s12967-017-1378-2
168. Raverdeau M, Cunningham SP, Harmon C, Lynch L. $\gamma\delta$ T cells in cancer: a small population of lymphocytes with big implications. *Clin Transl Immunology*. 2019;8(10):e01080. doi:10.1002/cti2.1080
169. Silva-Santos B, Serre K, Norell H. $\gamma\delta$ T cells in cancer. *Nat Rev Immunol*. Nov 2015;15(11):683-91. doi:10.1038/nri3904
170. Sen DR, Kaminski J, Barnitz RA, et al. The epigenetic landscape of T cell exhaustion. *Science*. 12 2016;354(6316):1165-1169. doi:10.1126/science.aae0491
171. Xia A, Zhang Y, Xu J, Yin T, Lu XJ. T Cell Dysfunction in Cancer Immunity and Immunotherapy. *Front Immunol*. 2019;10:1719. doi:10.3389/fimmu.2019.01719
172. Kurtulus S, Madi A, Escobar G, et al. Checkpoint Blockade Immunotherapy Induces Dynamic Changes in PD-1. *Immunity*. 01 2019;50(1):181-194.e6. doi:10.1016/j.immuni.2018.11.014
173. Im SJ, Hashimoto M, Gerner MY, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature*. 09 2016;537(7620):417-421. doi:10.1038/nature19330
174. Doedens AL, Phan AT, Stradner MH, et al. Hypoxia-inducible factors enhance the effector responses of CD8(+) T cells to persistent antigen. *Nat Immunol*. Nov 2013;14(11):1173-82. doi:10.1038/ni.2714

175. Tosolini M, Kirilovsky A, Mlecnik B, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res.* Feb 2011;71(4):1263-71. doi:10.1158/0008-5472.CAN-10-2907
176. Thorsson V, Gibbs DL, Brown SD, et al. The Immune Landscape of Cancer. *Immunity.* 04 2018;48(4):812-830.e14. doi:10.1016/j.immuni.2018.03.023
177. Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med.* 04 2017;9(1):34. doi:10.1186/s13073-017-0424-2
178. Kloor M, von Knebel Doeberitz M. The Immune Biology of Microsatellite-Unstable Cancer. *Trends Cancer.* 03 2016;2(3):121-133. doi:10.1016/j.trecan.2016.02.004
179. Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology.* Jun 2010;138(6):2073-2087.e3. doi:10.1053/j.gastro.2009.12.064
180. Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov.* Jan 2015;5(1):43-51. doi:10.1158/2159-8290.CD-14-0863
181. Pagès F, André T, Taieb J, et al. Prognostic and predictive value of the Immunoscore in stage III colon cancer patients treated with oxaliplatin in the prospective IDEA France PRODIGE-GERCOR cohort study. *Ann Oncol.* 07 2020;31(7):921-929. doi:10.1016/j.annonc.2020.03.310
182. Malesci A, Laghi L, Bianchi P, et al. Reduced likelihood of metastases in patients with microsatellite-unstable colorectal cancer. *Clin Cancer Res.* Jul 2007;13(13):3831-9. doi:10.1158/1078-0432.CCR-07-0366
183. Kloor M, Michel S, Buckowitz B, et al. Beta2-microglobulin mutations in microsatellite unstable colorectal tumors. *Int J Cancer.* Jul 2007;121(2):454-8. doi:10.1002/ijc.22691
184. Barrow P, Richman SD, Wallace AJ, et al. Confirmation that somatic mutations of beta-2 microglobulin correlate with a lack of recurrence in a subset of stage II mismatch repair deficient colorectal cancers from the QUASAR trial. *Histopathology.* Aug 2019;75(2):236-246. doi:10.1111/his.13895
185. Tikidzhieva A, Benner A, Michel S, et al. Microsatellite instability and Beta2-Microglobulin mutations as prognostic markers in colon cancer: results of the FOGT-4 trial. *Br J Cancer.* Mar 2012;106(6):1239-45. doi:10.1038/bjc.2012.53
186. Elliott JM, Wahle JA, Yokoyama WM. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med.* Sep 2010;207(10):2073-9. doi:10.1084/jem.20100986
187. Kärre K. Natural killer cell recognition of missing self. *Nat Immunol.* May 2008;9(5):477-80. doi:10.1038/ni0508-477
188. Shia J, Ellis NA, Paty PB, et al. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. *Am J Surg Pathol.* Nov 2003;27(11):1407-17. doi:10.1097/00000478-200311000-00002

189. Jass JR, Walsh MD, Barker M, Simms LA, Young J, Leggett BA. Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. *Eur J Cancer*. May 2002;38(7):858-66. doi:10.1016/s0959-8049(02)00041-2
190. Bohaumilitzky L, von Knebel Doeberitz M, Kloor M, Ahadova A. Implications of Hereditary Origin on the Immune Phenotype of Mismatch Repair-Deficient Cancers: Systematic Literature Review. *J Clin Med*. Jun 2020;9(6)doi:10.3390/jcm9061741
191. Schwitalle Y, Kloor M, Eiermann S, et al. Immune response against frameshift-induced neopeptides in HNPCC patients and healthy HNPCC mutation carriers. *Gastroenterology*. Apr 2008;134(4):988-97. doi:10.1053/j.gastro.2008.01.015
192. Kloor M, Reuschenbach M, Pauligk C, et al. A Frameshift Peptide Neoantigen-Based Vaccine for Mismatch Repair-Deficient Cancers: A Phase I/IIa Clinical Trial. *Clin Cancer Res*. Sep 2020;26(17):4503-4510. doi:10.1158/1078-0432.CCR-19-3517
193. Kang DH, Weaver MT, Park NJ, Smith B, McArdle T, Carpenter J. Significant impairment in immune recovery after cancer treatment. *Nurs Res*. 2009 Mar-Apr 2009;58(2):105-14. doi:10.1097/NNR.0b013e31818fced
194. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ*. Jan 2014;21(1):15-25. doi:10.1038/cdd.2013.67
195. Ramakrishnan R, Gabrilovich DI. Novel mechanism of synergistic effects of conventional chemotherapy and immune therapy of cancer. *Cancer Immunol Immunother*. Mar 2013;62(3):405-10. doi:10.1007/s00262-012-1390-6
196. Vacchelli E, Galluzzi L, Fridman WH, et al. Trial watch: Chemotherapy with immunogenic cell death inducers. *Oncoimmunology*. Mar 2012;1(2):179-188. doi:10.4161/onci.1.2.19026
197. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol*. 2013;31:51-72. doi:10.1146/annurev-immunol-032712-100008
198. Chajon E, Castelli J, Marsiglia H, De Crevoisier R. The synergistic effect of radiotherapy and immunotherapy: A promising but not simple partnership. *Crit Rev Oncol Hematol*. Mar 2017;111:124-132. doi:10.1016/j.critrevonc.2017.01.017
199. MOLE RH. Whole body irradiation; radiobiology or medicine? *Br J Radiol*. May 1953;26(305):234-41. doi:10.1259/0007-1285-26-305-234
200. Zaborowski AM, Winter DC, Lynch L. The therapeutic and prognostic implications of immunobiology in colorectal cancer: a review. *Br J Cancer*. Nov 2021;125(10):1341-1349. doi:10.1038/s41416-021-01475-x
201. Paschke S, Jafarov S, Staib L, et al. Are Colon and Rectal Cancer Two Different Tumor Entities? A Proposal to Abandon the Term Colorectal Cancer. *Int J Mol Sci*. Aug 2018;19(9)doi:10.3390/ijms19092577

202. Imperial R, Ahmed Z, Toor OM, et al. Comparative proteogenomic analysis of right-sided colon cancer, left-sided colon cancer and rectal cancer reveals distinct mutational profiles. *Mol Cancer*. 12 2018;17(1):177. doi:10.1186/s12943-018-0923-9
203. Teng F, Mu D, Meng X, et al. Tumor infiltrating lymphocytes (TILs) before and after neoadjuvant chemoradiotherapy and its clinical utility for rectal cancer. *Am J Cancer Res*. 2015;5(6):2064-74.
204. Shinto E, Hase K, Hashiguchi Y, et al. CD8+ and FOXP3+ tumor-infiltrating T cells before and after chemoradiotherapy for rectal cancer. *Ann Surg Oncol*. Jun 2014;21 Suppl 3:S414-21. doi:10.1245/s10434-014-3584-y
205. Matsutani S, Shibutani M, Maeda K, et al. Significance of tumor-infiltrating lymphocytes before and after neoadjuvant therapy for rectal cancer. *Cancer Sci*. Apr 2018;109(4):966-979. doi:10.1111/cas.13542
206. Anitei MG, Zeitoun G, Mlecnik B, et al. Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clin Cancer Res*. Apr 2014;20(7):1891-9. doi:10.1158/1078-0432.CCR-13-2830
207. Yasuda K, Nirei T, Sunami E, Nagawa H, Kitayama J. Density of CD4(+) and CD8(+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. *Radiat Oncol*. May 2011;6:49. doi:10.1186/1748-717X-6-49
208. Alawawdeh A, Krishnan T, Roy A, et al. Curative therapy for rectal cancer. *Expert Rev Anticancer Ther*. Feb 2021;21(2):193-203. doi:10.1080/14737140.2021.1845145
209. Bahadoer RR, Dijkstra EA, van Etten B, et al. Short-course radiotherapy followed by chemotherapy before total mesorectal excision (TME) versus preoperative chemoradiotherapy, TME, and optional adjuvant chemotherapy in locally advanced rectal cancer (RAPIDO): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 01 2021;22(1):29-42. doi:10.1016/S1470-2045(20)30555-6
210. Conroy T, Lamfichekh N, Etienne PL, et al. Total neoadjuvant therapy with mFOLFIRINOX versus preoperative chemoradiation in patients with locally advanced rectal cancer: Final results of PRODIGE 23 phase III trial, a -UNICANCER GI trial. ASCO20 Virtual Scientific Program. Abstract 4007 .
211. Kasi A, Abbasi S, Handa S, et al. Total Neoadjuvant Therapy vs Standard Therapy in Locally Advanced Rectal Cancer: A Systematic Review and Meta-analysis. *JAMA Netw Open*. 12 2020;3(12):e2030097. doi:10.1001/jamanetworkopen.2020.30097
212. Roxburgh CS, Shia J, Vakiani E, Daniel T, Weiser MR. Potential immune priming of the tumor microenvironment with FOLFOX chemotherapy in locally advanced rectal cancer. *Oncoimmunology*. 2018;7(6):e1435227. doi:10.1080/2162402X.2018.1435227
213. Mlecnik B, Bifulco C, Bindea G, et al. Multicenter International Society for Immunotherapy of Cancer Study of the Consensus Immunoscore for the Prediction of Survival and Response to Chemotherapy in Stage III Colon Cancer. *J Clin Oncol*. Nov 2020;38(31):3638-3651. doi:10.1200/JCO.19.03205

214. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell*. Apr 2015;27(4):450-61. doi:10.1016/j.ccell.2015.03.001
215. Yost KE, Satpathy AT, Wells DK, et al. Clonal replacement of tumor-specific T cells following PD-1 blockade. *Nat Med*. 08 2019;25(8):1251-1259. doi:10.1038/s41591-019-0522-3
216. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. 07 2017;357(6349):409-413. doi:10.1126/science.aan6733
217. Overman MJ, McDermott R, Leach JL, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol*. 09 2017;18(9):1182-1191. doi:10.1016/S1470-2045(17)30422-9
218. Chalabi M, Fanchi LF, Dijkstra KK, et al. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nat Med*. Apr 2020;doi:10.1038/s41591-020-0805-8
219. Malvezzi M, Carioli G, Bertuccio P, et al. European cancer mortality predictions for the year 2018 with focus on colorectal cancer. *Ann Oncol*. Apr 2018;29(4):1016-1022. doi:10.1093/annonc/mdy033
220. McKay A, Donaleshen J, Helewa RM, et al. Does young age influence the prognosis of colorectal cancer: a population-based analysis. *World J Surg Oncol*. Dec 2014;12:370. doi:10.1186/1477-7819-12-370
221. You YN, Dozois EJ, Boardman LA, Aakre J, Huebner M, Larson DW. Young-onset rectal cancer: presentation, pattern of care and long-term oncologic outcomes compared to a matched older-onset cohort. *Ann Surg Oncol*. Sep 2011;18(9):2469-76. doi:10.1245/s10434-011-1674-7
222. Orsini RG, Verhoeven RH, Lemmens VE, et al. Comparable survival for young rectal cancer patients, despite unfavourable morphology and more advanced-stage disease. *Eur J Cancer*. Sep 2015;51(13):1675-82. doi:10.1016/j.ejca.2015.06.005
223. Rodriguez L, Brennan K, Karim S, Nanji S, Patel SV, Booth CM. Disease Characteristics, Clinical Management, and Outcomes of Young Patients With Colon Cancer: A Population-based Study. *Clin Colorectal Cancer*. 12 2018;17(4):e651-e661. doi:10.1016/j.clcc.2018.06.007
224. Abdelsattar ZM, Wong SL, Regenbogen SE, Jomaa DM, Hardiman KM, Hendren S. Colorectal cancer outcomes and treatment patterns in patients too young for average-risk screening. *Cancer*. Mar 2016;122(6):929-34. doi:10.1002/cnccr.29716
225. Ryan R, Gibbons D, Hyland JM, et al. Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *Histopathology*. Aug 2005;47(2):141-6. doi:10.1111/j.1365-2559.2005.02176.x
226. Siegel RL, Fedewa SA, Anderson WF, et al. Colorectal Cancer Incidence Patterns in the United States, 1974-2013. *J Natl Cancer Inst*. 08 2017;109(8)doi:10.1093/jnci/djw322

227. Wang MJ, Ping J, Li Y, et al. The prognostic factors and multiple biomarkers in young patients with colorectal cancer. *Sci Rep*. May 2015;5:10645. doi:10.1038/srep10645
228. Group GTS. Prolongation of the disease-free interval in surgically treated rectal carcinoma. *N Engl J Med*. 06 1985;312(23):1465-72. doi:10.1056/NEJM198506063122301
229. Krook JE, Moertel CG, Gunderson LL, et al. Effective surgical adjuvant therapy for high-risk rectal carcinoma. *N Engl J Med*. Mar 1991;324(11):709-15. doi:10.1056/NEJM199103143241101
230. Petersen SH, Harling H, Kirkeby LT, Wille-Jørgensen P, Mocellin S. Postoperative adjuvant chemotherapy in rectal cancer operated for cure. *Cochrane Database Syst Rev*. Mar 2012;(3):CD004078. doi:10.1002/14651858.CD004078.pub2
231. Peterse EFP, Meester RGS, Siegel RL, et al. The impact of the rising colorectal cancer incidence in young adults on the optimal age to start screening: Microsimulation analysis I to inform the American Cancer Society colorectal cancer screening guideline. *Cancer*. Jul 2018;124(14):2964-2973. doi:10.1002/cncr.31543
232. Wolf AMD, Fontham ETH, Church TR, et al. Colorectal cancer screening for average-risk adults: 2018 guideline update from the American Cancer Society. *CA Cancer J Clin*. 07 2018;68(4):250-281. doi:10.3322/caac.21457
233. Brenner DR, Heer E, Sutherland RL, et al. National Trends in Colorectal Cancer Incidence Among Older and Younger Adults in Canada. *JAMA Netw Open*. 07 2019;2(7):e198090. doi:10.1001/jamanetworkopen.2019.8090
234. Zaborowski AM, Abdile A, Adamina M, et al. Characteristics of Early-Onset vs Late-Onset Colorectal Cancer: A Review. *JAMA Surg*. Sep 01 2021;156(9):865-874. doi:10.1001/jamasurg.2021.2380
235. Yeo SA, Chew MH, Koh PK, Tang CL. Young colorectal carcinoma patients do not have a poorer prognosis: a comparative review of 2,426 cases. *Tech Coloproctol*. Dec 2013;17(6):653-61. doi:10.1007/s10151-013-0977-z
236. Yantiss RK, Goodarzi M, Zhou XK, et al. Clinical, pathologic, and molecular features of early-onset colorectal carcinoma. *Am J Surg Pathol*. Apr 2009;33(4):572-82. doi:10.1097/PAS.0b013e31818afd6b
237. Mueller M, Schneider MA, Deplazes B, Cabalzar-Wondberg D, Rickenbacher A, Turina M. Colorectal cancer of the young displays distinct features of aggressive tumor biology: A single-center cohort study. *World J Gastrointest Surg*. Feb 2021;13(2):164-175. doi:10.4240/wjgs.v13.i2.164
238. Quah HM, Joseph R, Schrag D, et al. Young age influences treatment but not outcome of colon cancer. *Ann Surg Oncol*. Oct 2007;14(10):2759-65. doi:10.1245/s10434-007-9465-x
239. McMillan DC, McArdle CS. The impact of young age on cancer-specific and non-cancer-related survival after surgery for colorectal cancer: 10-year follow-up. *Br J Cancer*. Aug 2009;101(4):557-60. doi:10.1038/sj.bjc.6605222
240. Kozak VN, Kalady MF, Gamaleldin MM, Liang J, Church JM. Colorectal surveillance after segmental resection for young-onset colorectal cancer: is there evidence for extended resection? *Colorectal Dis*. Nov 2017;19(11):O386-O392. doi:10.1111/codi.13874

241. Klos CL, Montenegro G, Jamal N, et al. Segmental versus extended resection for sporadic colorectal cancer in young patients. *J Surg Oncol*. Sep 2014;110(3):328-32. doi:10.1002/jso.23649
242. Manjelievskaia J, Brown D, McGlynn KA, Anderson W, Shriver CD, Zhu K. Chemotherapy Use and Survival Among Young and Middle-Aged Patients With Colon Cancer. *JAMA Surg*. 05 2017;152(5):452-459. doi:10.1001/jamasurg.2016.5050
243. Le DT, Kim TW, Van Cutsem E, et al. Phase II Open-Label Study of Pembrolizumab in Treatment-Refractory, Microsatellite Instability-High/Mismatch Repair-Deficient Metastatic Colorectal Cancer: KEYNOTE-164. *J Clin Oncol*. 01 2020;38(1):11-19. doi:10.1200/JCO.19.02107
244. Mohan HM, Ryan E, Balasubramanian I, et al. Microsatellite instability is associated with reduced disease specific survival in stage III colon cancer. *Eur J Surg Oncol*. Nov 2016;42(11):1680-1686. doi:10.1016/j.ejso.2016.05.013
245. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol*. Jul 2010;28(20):3219-26. doi:10.1200/JCO.2009.27.1825
246. Sinicrope FA, Foster NR, Thibodeau SN, et al. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst*. Jun 2011;103(11):863-75. doi:10.1093/jnci/djr153
247. Ryan É, Khaw YL, Creavin B, et al. Tumor Budding and PDC Grade Are Stage Independent Predictors of Clinical Outcome in Mismatch Repair Deficient Colorectal Cancer. *Am J Surg Pathol*. Jan 2018;42(1):60-68. doi:10.1097/PAS.0000000000000931
248. Lugli A, Vljajnic T, Giger O, et al. Intratumoral budding as a potential parameter of tumor progression in mismatch repair-proficient and mismatch repair-deficient colorectal cancer patients. *Hum Pathol*. Dec 2011;42(12):1833-40. doi:10.1016/j.humpath.2011.02.010
249. Guastadisegni C, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. *Eur J Cancer*. Oct 2010;46(15):2788-98. doi:10.1016/j.ejca.2010.05.009
250. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol*. Jan 2005;23(3):609-18. doi:10.1200/JCO.2005.01.086
251. Samowitz WS, Curtin K, Ma KN, et al. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiol Biomarkers Prev*. Sep 2001;10(9):917-23.
252. Petrelli F, Ghidini M, Cabiddu M, et al. Microsatellite Instability and Survival in Stage II Colorectal Cancer: A Systematic Review and Meta-analysis. *Anticancer Res*. Dec 2019;39(12):6431-6441. doi:10.21873/anticancer.13857
253. Merok MA, Ahlquist T, Røyrvik EC, et al. Microsatellite instability has a positive prognostic impact on stage II colorectal cancer after complete resection: results from a large, consecutive

Norwegian series. *Ann Oncol*. May 2013;24(5):1274-82. doi:10.1093/annonc/mds614

254. Reticker-Flynn NE, Zhang W, Belk JA, et al. Lymph node colonization induces tumor-immune tolerance to promote distant metastasis. *Cell*. 05 26 2022;185(11):1924-1942.e23. doi:10.1016/j.cell.2022.04.019

255. Hernandez-Sanchez A, Grossman M, Yeung K, Sei SS, Lipkin S, Kloor M. Vaccines for immunoprevention of DNA mismatch repair deficient cancers. *J Immunother Cancer*. 06 2022;10(6)doi:10.1136/jitc-2021-004416

256. Kim CG, Ahn JB, Jung M, et al. Effects of microsatellite instability on recurrence patterns and outcomes in colorectal cancers. *Br J Cancer*. 06 28 2016;115(1):25-33. doi:10.1038/bjc.2016.161

257. Cercek A, Dos Santos Fernandes G, Roxburgh CS, et al. Mismatch Repair-Deficient Rectal Cancer and Resistance to Neoadjuvant Chemotherapy. *Clin Cancer Res*. 07 01 2020;26(13):3271-3279. doi:10.1158/1078-0432.CCR-19-3728

258. de Rosa N, Rodriguez-Bigas MA, Chang GJ, et al. DNA Mismatch Repair Deficiency in Rectal Cancer: Benchmarking Its Impact on Prognosis, Neoadjuvant Response Prediction, and Clinical Cancer Genetics. *J Clin Oncol*. 09 2016;34(25):3039-46. doi:10.1200/JCO.2016.66.6826

259. O'Connell E, Reynolds IS, McNamara DA, Prehn JHM, Burke JP. Microsatellite instability and response to neoadjuvant chemoradiotherapy in rectal cancer: A systematic review and meta-analysis. *Surg Oncol*. Sep 2020;34:57-62. doi:10.1016/j.suronc.2020.03.009

260. Denost Q. The challenge posed by young-onset rectal cancer. *Br J Surg*. 04 2020;107(5):481-483. doi:10.1002/bjs.11591

261. Sun M, Wang Y, Sundquist J, Sundquist K, Ji J. Temporal Trends of Sex Disparity in Incidence and Survival of Colorectal Cancer: Variations by Anatomical Site and Age at Diagnosis. *Clin Epidemiol*. 2020;12:73-81. doi:10.2147/CLEP.S240006

262. Haak HE, Beets GL, Peeters K, et al. Prevalence of nodal involvement in rectal cancer after chemoradiotherapy. *Br J Surg*. Oct 23 2021;108(10):1251-1258. doi:10.1093/bjs/znab194

263. van der Valk MJM, Hilling DE, Bastiaannet E, et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. *Lancet*. 06 2018;391(10139):2537-2545. doi:10.1016/S0140-6736(18)31078-X

264. Bahadoer RR, Peeters KCMJ, Beets GL, et al. Watch and wait after a clinical complete response in rectal cancer patients younger than 50 years. *Br J Surg*. 12 17 2021;109(1):114-120. doi:10.1093/bjs/znab372

265. Fernandez LM, São Julião GP, Figueiredo NL, et al. Conditional recurrence-free survival of clinical complete responders managed by watch and wait after neoadjuvant chemoradiotherapy for rectal cancer in the International Watch & Wait Database: a retrospective, international, multicentre registry study. *Lancet Oncol*. 01 2021;22(1):43-50. doi:10.1016/S1470-2045(20)30557-X

266. Renehan AG, Malcomson L, Emsley R, et al. Watch-and-wait approach versus surgical resection after chemoradiotherapy for patients with rectal cancer (the OnCoRe project): a propensity-score matched cohort analysis. *Lancet Oncol*. Feb 2016;17(2):174-183. doi:10.1016/S1470-2045(15)00467-2
267. Gani C, Gani N, Zschaecck S, et al. Organ Preservation in Rectal Cancer: The Patients' Perspective. *Front Oncol*. 2019;9:318. doi:10.3389/fonc.2019.00318
268. Kennedy ED, Borowiec AM, Schmocker S, et al. Patient and Physician Preferences for Nonoperative Management for Low Rectal Cancer: Is It a Reasonable Treatment Option? *Dis Colon Rectum*. 11 2018;61(11):1281-1289. doi:10.1097/DCR.0000000000001166
269. Noordman BJ, de Bekker-Grob EW, Coene PPL, et al. Patients' preferences for treatment after neoadjuvant chemoradiotherapy for oesophageal cancer. *Br J Surg*. 11 2018;105(12):1630-1638. doi:10.1002/bjs.10897
270. Segelman J, Buchli C, Svanström R, et al. Effect of radiotherapy for rectal cancer on ovarian androgen production. *Br J Surg*. 02 2019;106(3):267-275. doi:10.1002/bjs.10980
271. Kang YM, Chao TF, Wang TH, Hu YW. Increased risk of pelvic fracture after radiotherapy in rectal cancer survivors: A propensity matched study. *Cancer Med*. 07 2019;8(8):3639-3647. doi:10.1002/cam4.2030
272. Mols F, Beijers T, Lemmens V, van den Hurk CJ, Vreugdenhil G, van de Poll-Franse LV. Chemotherapy-induced neuropathy and its association with quality of life among 2- to 11-year colorectal cancer survivors: results from the population-based PROFILES registry. *J Clin Oncol*. Jul 20 2013;31(21):2699-707. doi:10.1200/JCO.2013.49.1514
273. Healey Bird BRJ. Total neoadjuvant therapy for locally advanced rectal cancer: the fuse is lit. *Br J Surg*. 12 2020;107(13):1705-1707. doi:10.1002/bjs.12014
274. Chauvin JM, Pagliano O, Fourcade J, et al. TIGIT and PD-1 impair tumor antigen-specific CD8⁺ T cells in melanoma patients. *J Clin Invest*. May 2015;125(5):2046-58. doi:10.1172/JCI80445
275. Chauvin JM, Zarour HM. TIGIT in cancer immunotherapy. *J Immunother Cancer*. 09 2020;8(2)doi:10.1136/jitc-2020-000957
276. Hu H, Kang L, Zhang J, et al. Neoadjuvant PD-1 blockade with toripalimab, with or without celecoxib, in mismatch repair-deficient or microsatellite instability-high, locally advanced, colorectal cancer (PICC): a single-centre, parallel-group, non-comparative, randomised, phase 2 trial. *Lancet Gastroenterol Hepatol*. 01 2022;7(1):38-48. doi:10.1016/S2468-1253(21)00348-4
277. Diaz LA, Shiu KK, Kim TW, et al. Pembrolizumab versus chemotherapy for microsatellite instability-high or mismatch repair-deficient metastatic colorectal cancer (KEYNOTE-177): final analysis of a randomised, open-label, phase 3 study. *Lancet Oncol*. 05 2022;23(5):659-670. doi:10.1016/S1470-2045(22)00197-8
278. Andre T, Amonkar M, Norquist JM, et al. Health-related quality of life in patients with microsatellite instability-high or mismatch repair deficient metastatic colorectal cancer treated with first-line pembrolizumab versus chemotherapy (KEYNOTE-177): an

open-label, randomised, phase 3 trial. *Lancet Oncol.* 05 2021;22(5):665-677. doi:10.1016/S1470-2045(21)00064-4

279. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature.* Nov 2014;515(7528):568-71. doi:10.1038/nature13954

280. Simon S, Labarriere N. PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *Oncoimmunology.* 2017 2017;7(1):e1364828. doi:10.1080/2162402X.2017.1364828

281. Giannakis M, Mu XJ, Shukla SA, et al. Genomic Correlates of Immune-Cell Infiltrates in Colorectal Carcinoma. *Cell Rep.* Apr 2016;15(4):857-865. doi:10.1016/j.celrep.2016.03.075

282. Domingo E, Freeman-Mills L, Rayner E, et al. Somatic POLE proofreading domain mutation, immune response, and prognosis in colorectal cancer: a retrospective, pooled biomarker study. *Lancet Gastroenterol Hepatol.* 11 2016;1(3):207-216. doi:10.1016/S2468-1253(16)30014-0

283. Guerra J, Pinto C, Pinto D, et al. POLE somatic mutations in advanced colorectal cancer. *Cancer Med.* Dec 2017;6(12):2966-2971. doi:10.1002/cam4.1245

284. Gong J, Wang C, Lee PP, Chu P, Fakih M. Response to PD-1 Blockade in Microsatellite Stable Metastatic Colorectal Cancer Harboring a. *J Natl Compr Canc Netw.* 02 2017;15(2):142-147. doi:10.6004/jnccn.2017.0016

285. Ishikawa T, Fujita T, Suzuki Y, et al. Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. *Cancer Res.* Sep 2003;63(17):5564-72.

286. Angelova M, Charoentong P, Hackl H, et al. Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome Biol.* Mar 2015;16:64. doi:10.1186/s13059-015-0620-6

287. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med.* Aug 2015;21(8):938-945. doi:10.1038/nm.3909

288. Ribot JC, Lopes N, Silva-Santos B. $\gamma\delta$ T cells in tissue physiology and surveillance. *Nat Rev Immunol.* 04 2021;21(4):221-232. doi:10.1038/s41577-020-00452-4

289. Ma R, Yuan D, Guo Y, Yan R, Li K. Immune Effects of $\gamma\delta$ T Cells in Colorectal Cancer: A Review. *Front Immunol.* 2020;11:1600. doi:10.3389/fimmu.2020.01600

290. Wu P, Wu D, Ni C, et al. $\gamma\delta$ T17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity.* May 2014;40(5):785-800. doi:10.1016/j.immuni.2014.03.013

291. Meraviglia S, Lo Presti E, Tosolini M, et al. Distinctive features of tumor-infiltrating $\gamma\delta$ T lymphocytes in human colorectal cancer. *Oncoimmunology.* 2017 2017;6(10):e1347742. doi:10.1080/2162402X.2017.1347742

292. Li XD, Miao SY, Wang GL, Yang L, Shu YQ, Yin YM. Amphiregulin and epiregulin expression in colorectal carcinoma and

- the correlation with clinicopathological characteristics. *Onkologie*. 2010;33(7):353-8. doi:10.1159/000315380
293. Krasinskas AM. EGFR Signaling in Colorectal Carcinoma. *Patholog Res Int*. Feb 2011;2011:932932. doi:10.4061/2011/932932
294. Ohchi T, Akagi Y, Kinugasa T, et al. Amphiregulin is a prognostic factor in colorectal cancer. *Anticancer Res*. Jun 2012;32(6):2315-21.
295. Stahler A, Stintzing S, Modest DP, et al. Amphiregulin Expression Is a Predictive Biomarker for. *Clin Cancer Res*. 12 2020;26(24):6559-6567. doi:10.1158/1078-0432.CCR-20-2748
296. Mayassi T, Ladell K, Gudjonson H, et al. Chronic Inflammation Permanently Reshapes Tissue-Resident Immunity in Celiac Disease. *Cell*. 02 21 2019;176(5):967-981.e19. doi:10.1016/j.cell.2018.12.039
297. Marin-Acevedo JA, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. *J Hematol Oncol*. 03 15 2018;11(1):39. doi:10.1186/s13045-018-0582-8
298. Harjunpää H, Guillerey C. TIGIT as an emerging immune checkpoint. *Clin Exp Immunol*. 05 2020;200(2):108-119. doi:10.1111/cei.13407
299. Ge Z, Peppelenbosch MP, Sprengers D, Kwekkeboom J. TIGIT, the Next Step Towards Successful Combination Immune Checkpoint Therapy in Cancer. *Front Immunol*. 2021;12:699895. doi:10.3389/fimmu.2021.699895
300. Pizzolato G, Kaminski H, Tosolini M, et al. Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRV δ 1 and TCRV δ 2 $\gamma\delta$ T lymphocytes. *Proc Natl Acad Sci U S A*. 06 11 2019;116(24):11906-11915. doi:10.1073/pnas.1818488116
301. Kuttruff S, Koch S, Kelp A, Pawelec G, Rammensee HG, Steinle A. NKp80 defines and stimulates a reactive subset of CD8 T cells. *Blood*. Jan 08 2009;113(2):358-69. doi:10.1182/blood-2008-03-145615
302. Freud AG, Keller KA, Scoville SD, et al. NKp80 Defines a Critical Step during Human Natural Killer Cell Development. *Cell Rep*. 07 12 2016;16(2):379-391. doi:10.1016/j.celrep.2016.05.095
303. Cercek A, Lumish M, Sinopoli J, et al. PD-1 Blockade in Mismatch Repair-Deficient, Locally Advanced Rectal Cancer. *N Engl J Med*. 06 23 2022;386(25):2363-2376. doi:10.1056/NEJMoa2201445
304. Jang TH, Park SC, Yang JH, et al. Cryopreservation and its clinical applications. *Integr Med Res*. Mar 2017;6(1):12-18. doi:10.1016/j.imr.2016.12.001
305. Wang SY, Hsu ML, Tzeng CH, Hsu HC, Ho CK. The influence of cryopreservation on cytokine production by human T lymphocytes. *Cryobiology*. Aug 1998;37(1):22-9. doi:10.1006/cryo.1998.2094
306. Fujiwara S, Akiyama M, Yamakido M, et al. Cryopreservation of human lymphocytes for assessment of lymphocyte subsets and natural killer cytotoxicity. *J Immunol Methods*. Jun 24 1986;90(2):265-73. doi:10.1016/0022-1759(86)90084-0

307. Zaborowski AM, Stakelum A, Winter DC. Anastomotic leak risk in complete responders to neoadjuvant therapy for rectal cancer: a systematic review. *Int J Colorectal Dis.* Apr 2021;36(4):671-676. doi:10.1007/s00384-021-03833-w
308. Bailey CE, Tran Cao HS, Hu CY, et al. Functional deficits and symptoms of long-term survivors of colorectal cancer treated by multimodality therapy differ by age at diagnosis. *J Gastrointest Surg.* Jan 2015;19(1):180-8; discussion 188. doi:10.1007/s11605-014-2645-7