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Les cancers colorectaux sont majoritairement microsatellites stables et peu sensibles à l'immunothérapie. La radiothérapie (RT), par ses effets immunomodulateurs, pourrait sensibiliser ces tumeurs à l'immunothérapie. Le traitement néoadjuvant total assure un bon contrôle local et à distance dans le cancer rectal localement avancé. Le pronostic reste défavorable dans les formes métastatiques. Ce travail visait à caractériser les mécanismes immunitaires induits par la RT chez l'humain et à optimiser les associations RT-immunothérapie à l'aide de techniques modernes de RT.

La RT adaptative guidée par cone-beam CT a été évaluée pour les tumeurs rectales et hépatiques. Une revue systématique des pratiques de boost rectal a démontré une forte hétérogénéité, soulignant le besoin de recommandations internationales. La RT préopératoire entraîne des effets immunitaires systémiques limités et active surtout des voies immunitaires intratumorales, sans modification majeure de l'infiltration immune.

Colorectal cancers are mostly microsatellite stable and poorly responsive to immunotherapy. Radiotherapy (RT), through its immunomodulatory effects, may sensitise these tumours to immunotherapy. Treatment goals differ by stage: total neoadjuvant therapy supports disease control and non-operative management in locally advanced rectal cancer, whereas prognosis remains poor in metastatic disease. This work aimed to characterise RT-induced immune mechanisms in humans and to optimise RT-immunotherapy combinations using modern RT techniques.

Dedicated cone-beam CT-guided online-adaptive RT workflows were developed for rectal cancer and liver stereotactic ablative RT. A systematic review of rectal boost strategies highlighted heterogeneous practices, supporting the need for international guidelines. Overall, preoperative RT showed limited systemic immune effects and predominantly activated intratumoral immune pathways rather than substantially modify immune cell infiltration.

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Julien Pierrard

Optimising radiotherapy for immune synergy in colorectal cancer

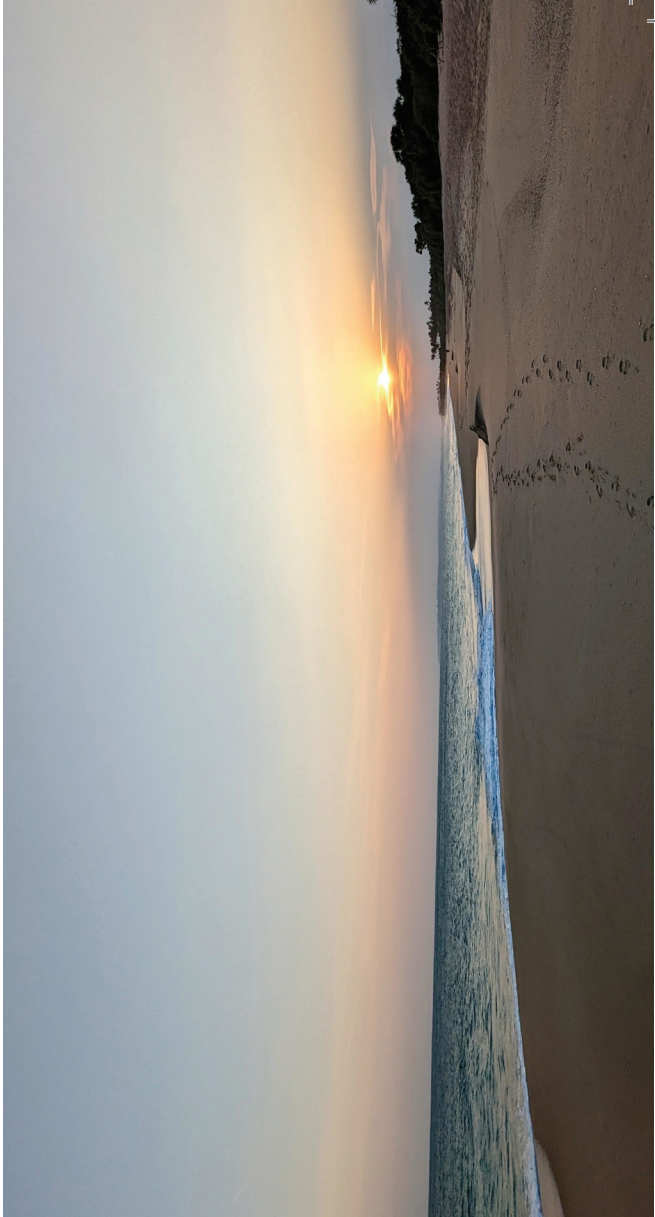
Optimising radiotherapy techniques for radiation-immune synergy in colorectal cancer

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Thèse présentée en vue de l'obtention du grade de docteur en Sciences médicales

Secteur des sciences de la santé



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## Summary

Colorectal cancer (CRC) is a major health burden. Most cases are microsatellite stable (MSS) and are poorly responsive to immunotherapy. Sensitising MSS CRC to immunotherapy is a major challenge, for which radiotherapy (RT) may be an option, given the local and systemic immunomodulatory effects demonstrated preclinically. Treatment goals differ by stage: in locally advanced rectal cancer (LARC), total neoadjuvant therapy (TNT) enables excellent local and distant control, allowing development of non-operative management strategies for quality of life improvement through organ preservation. In metastatic MSS CRC, overall survival remains poor due to limited therapeutic options. This work aimed to characterise RT-induced immune mechanisms in humans and to exploit this knowledge to optimise RT-immunotherapy combinations. Dedicated workflows using modern RT techniques were developed for LARC RT and liver stereotactic ablative RT (SABR).

In LARC, cone-beam CT (CBCT)-guided online-adaptive RT (oART) was assessed for primary tumour dose escalation. By reducing planning target volume (PTV) margins compared with non-adaptive RT, oART enabled escalation without increasing toxicity risk. As rectal boost is widely performed despite lacking recommendations, a systematic review of boost techniques was performed, revealing high heterogeneity. Meta-analysis identified RT parameters associated with pathological complete response. Then, a protocol was proposed, evaluating a new TNT strategy in which tumour-draining lymph node (TDLN) RT is delayed from primary tumour RT

to preserve the efficacy of immunotherapy given concomitantly. This aims to improve clinical complete response and to expand non-operative management eligibility. This proposed protocol should primarily be regarded as a basis for discussion, highlighting the multiple biological, technical, and clinical parameters that must be carefully considered when envisaging a combined radiotherapy–immunotherapy–chemotherapy strategy, rather than as a definitive therapeutic recommendation.

In metastatic CRC, CBCT-guided oART was explored to avoid invasive radiopaque marker implantation for liver SABR. While automatic propagation of target volumes on daily CBCT required larger PTV to compensate for propagation errors, daily adaptation improved target coverage and organ-at-risk sparing. Mechanically assisted non-invasive ventilation (MANIV) for breath-hold enhanced CBCT quality, reduced inter-operator image-guided RT variability, and improved autosegmentation accuracy. Finally, RT-induced immune effects were analysed by RT dose and site in MSS CRC patient. Pre- and post-RT blood comparison showed only an increase of a CD4<sup>+</sup> T cell subset. In tumours, analysis revealed activation of immune pathways in irradiated lesions rather than major changes in infiltrating immune cell composition.

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## List of abbreviations

BED: Biologically effective dose  
CBCT: Cone-beam computed tomography  
CRC: Colorectal cancer  
CT: Computed tomography  
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4  
CTV: Clinical target volume  
dMMR/MSI-H: deficient DNA mismatch repair microsatellite instability–high  
GTV: Gross tumour volume  
IFN: Interferon  
IMRT/VMAT: intensity-modulated radiotherapy/volumetric-modulated arctherapy  
LARC: Locally advanced rectal cancer  
MANIV(-BH): Mechanically assisted non-invasive ventilation(-breath hold)  
mFOLFIRINOX: modified 5-fluorouracil, irinotecan, and oxaliplatin  
MSS : microsatellite stable  
OAR: Organ-at-risk  
oART: Online-adaptive radiotherapy  
PD-(L)1: Programmed cell death(-ligand) 1  
PET-CT: Positron emission tomography-computed tomography  
PTV: Planning target volume  
SABR: Stereotactic ablative radiotherapy  
TDLN: Tumour-draining lymph node  
TGF: Transforming growth factor  
TME: Total mesorectal excision  
TNT: Total neoadjuvant therapy  
Treg: Regulatory T cell



# Chapter 1

## 1

# Introduction

## **1.1. Epidemiology of colorectal cancer**

### **1.1.1. Incidence and mortality**

In 2022, colorectal cancer (CRC) was the fourth common cancer worldwide, with an age-standardised incidence of 18.4 cases per 100 000 person-years, representing around 1.9 million new diagnoses each year. In Belgium, the incidence is markedly higher, at 29.2 per 100 000 person-years, 8.4 of which are rectal tumours [1].

CRC is also the second-leading cause of cancer death after lung cancer, responsible for about 905 000 deaths globally and roughly 3200 deaths annually in Belgium [1, 2].

CRC is therefore a significant public health care issue that is projected to grow even further over the next years [1, 2]. Indeed, an increased incidence of 22.6% by 2030 and 85.5% by 2050 is expected. Also, the mortality is expected to rise by 24.2% in 2030 and 103.2% in 2050 (Figure 1.1.1) [1].

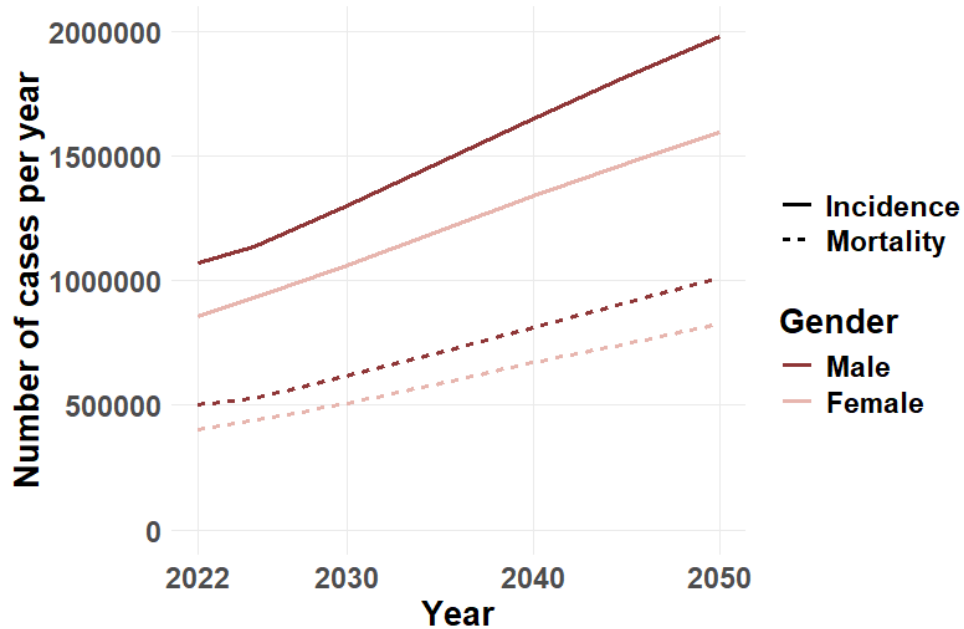


Figure 1.1.1: Projected trends in colorectal cancer incidence and mortality by sex through 2050. Adapted from World Health Organization, *Global cancer statistics 2022*.

### 1.1.2. Distribution

CRC predominantly affects older individuals, with approximately 85 % of cases diagnosed in people over the age of 50 [2, 3]. However, the incidence of early-onset CRC (diagnosed before age 50) is rising globally by 2 – 4% annually, likely driven by modification of lifestyle such as increased sedentary behaviour and poor dietary habits, which contribute to earlier onset of cancer-associated conditions like obesity, diabetes, and metabolic syndrome [4]. In contrast, CRC incidence in elderly is declining, due to smoking reduction and implementation of screening programmes [4, 5].



Men are 31% more likely to develop CRC than women (Figure 1.1) , and this disparity is even greater for rectal cancer, where the risk is 75 % higher in men [1-3, 6, 7]. This sex-based difference may be linked to lower alcohol and red meat consumption in women, differences in gut microbiota composition, and the protective effects of oestrogens [6].

Among ethnic groups, African Americans exhibit the highest CRC incidence, whereas Asians and Hispanics show the lowest rates [2, 3, 8]. These disparities may be explained by differences in environmental exposures, socioeconomic status, and genetic susceptibility [8].

Geographically, high-income countries have the highest CRC incidence, particularly in Oceania, followed by Western Europe and North America, while incidence rates are lowest in Africa and South Asia [1, 4, 9]. These variations reflect differences in dietary habits, environmental exposures, age demographics, and screening practice [4, 9]. Nevertheless, with the ongoing westernisation of lifestyles in developing regions, CRC incidence is increasing in areas such as East-Asia and South America (Figure 1.1.2) [4, 7, 9].

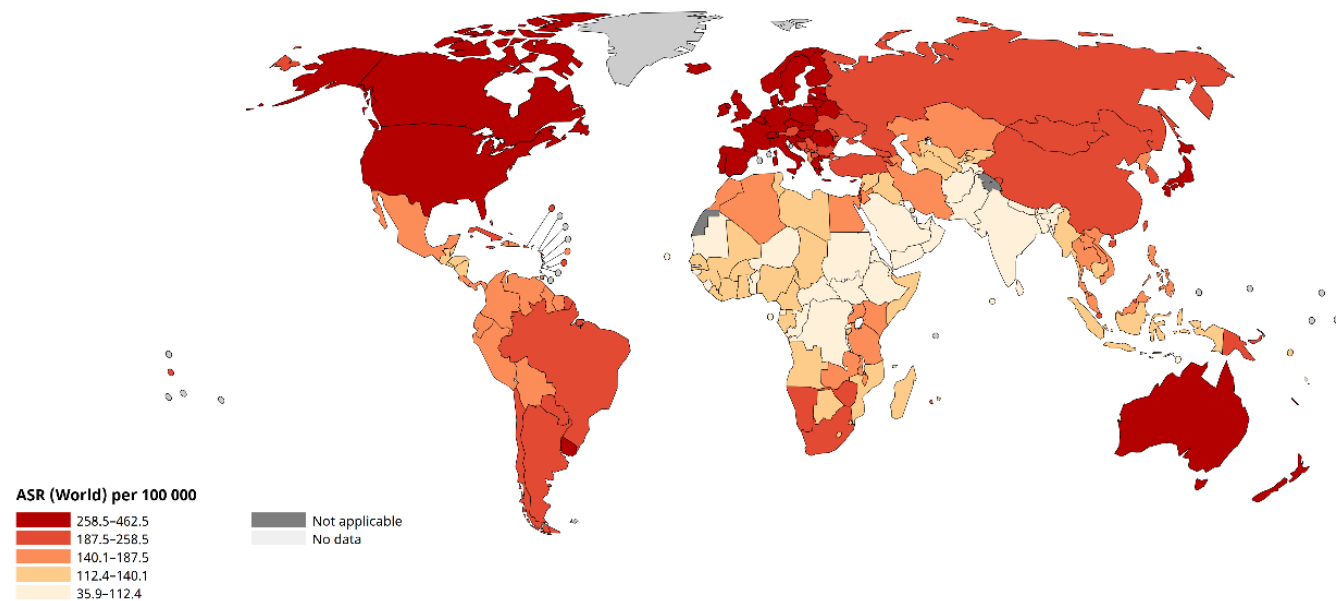


Figure 1.1.2: Colorectal cancer incidence by country. Adapted from World Health Organization, Global cancer statistics 2022. ASR: Age-standardised rate.

### **1.1.3. Risk factors**

Sporadic cancers account for roughly 75% of CRC. Their development is promoted by several risk factors:

- Lifestyle-related: high intake of red or ultra-processed meat, alcohol consumption, smoking, physical inactivity [10-12].
- Metabolic comorbidities: central obesity and type 2 diabetes mellitus [11, 12]
- Pre-existing gastrointestinal conditions: adenomas, Crohn's disease, ulcerative colitis, and previous abdominal or pelvic radiotherapy (RT) [7, 11-13]
- Non-modifiable: increasing age, male sex, and ethnic backgrounds [7, 12]

In contrast, regular physical activity and diets rich in fibre, fruit, and vegetables are protective, as are adequate calcium and vitamin D intake and, potentially, low-dose aspirin [10-12, 14, 15]

Non-syndromic familial predisposition explains about 25 % of CRC; having a first-degree relative with CRC roughly doubles an individual's risk [11, 12].

Hereditary cancer syndromes cause the remaining 5% [7]. Hereditary nonpolyposis colorectal cancer (mutation in DNA mismatch repair genes) including Lynch syndrome and familial adenomatous polyposis coli (*APC* mutation) are the two main causes of hereditary CRC. Less common entities include Turcot syndrome, Peutz-Jeghers (*STK11* mutation), and *MUTYH*-associated polyposis syndrome (*MUTYH* mutation) [7, 12].

## **1.2. Physiopathology of colorectal cancer**

### **1.2.1. Carcinogenesis**

As for other cancers, CRC follows the classical “initiation-promotion-progression-metastasis” model. During initiation, colorectal cells accumulate genetic mutations that predispose them to malignant transformation. Promotion involves enhanced cellular proliferation, while progression is marked by the acquisition of invasive and aggressive features. Metastasis, the final stage, corresponds to the spread of cancer cells to distant organs [16].

Three major genetic pathways leading to CRC have been identified, though they are not mutually exclusive and tumours often display overlapping features [17]. The conventional chromosomal instability (CIN) pathway, responsible for approximately 70% of CRCs, is a multistep process typically initiated by *APC* mutations, followed by mutations in *KRAS*, *PIK3CA* and *SMAD4*, loss of heterozygosity of chromosome 18, and *TP53* mutations [17-19]. The CpG island methylator phenotype (CIMP) pathway involves hypermethylation of promoter regions in tumour suppressor genes such as *MLH1*, *MINT1*, *MINT2* and *MINT3* [17, 18, 20]. The third pathway, characterised by high DNA microsatellite instability (dMMR/MSI-H), results from defects in MMR genes [17, 18, 21].

Phenotypic carcinogenic evolution can also be described through three main pathways, which may overlap with these genetic mechanisms. The adenoma-carcinoma sequence, accounting for 85-90% of sporadic CRCs, typically aligns with the conventional chromosomal instability pathway and

progresses from normal epithelium to early and late adenoma, and, finally, adenocarcinoma. The serrated pathway, responsible for 10 – 15% of sporadic CRCs, is frequently linked to CpG island hypermethylation and progresses from hyperplastic polyps to sessile serrated adenomas and then to adenocarcinoma. Lastly, the inflammatory pathway, driven by chronic inflammation such as in inflammatory bowel disease (<2% of CRCs), involves progression from indefinite to low- and high-grade dysplasia before transformation into adenocarcinoma [17, 18].

The "seed and soil" hypothesis proposes that metastatic tumour cells colonise specific organs based on both microenvironmental and anatomical compatibility [22]. The metastatic cascade begins with the epithelial-mesenchymal transition, during which tumour cells lose epithelial characteristics (polarity, adhesion) and acquire mesenchymal traits (motility, invasiveness) and immune evasion capacity [23]. These cells can then migrate to distant organs primed by tumour-derived factors to form pre-metastatic niche [24]. For more information about this pre-metastatic niche formation, see Appendix 1 [25].

In CRC, dissemination occurs via lymphatic, hematogenous, or transperitoneal routes. Lymphatic spread follows sequential lymph node stations, dictated by tumour location. The initial tumour-draining lymph nodes (TDLNs) are pericolic for colonic tumours and mesorectal for rectal tumours. Migration continues along major vascular axes: superior mesenteric (caecum, right and transverse colon), inferior mesenteric (transverse, left, sigmoid colon, and upper rectum), and internal iliac (mid

and lower rectum) [26]. Hematogenous metastases most frequently involve the liver and lungs, while bone and brain are less common sites [27]. Tumours of the colon and upper rectum drain into the portal system, favouring liver metastases, whereas mid and lower rectal tumours drain into the systemic circulation via the inferior vena cava, leading more commonly to lung metastases. The lungs may represent an initial metastatic site that facilitates subsequent dissemination to other organs, as brain metastases are more frequently associated with pulmonary than hepatic involvement [27]. Finally, due to proximity with the peritoneum, CRC can also spread via transcoelomic dissemination, particularly in mucinous or signet ring histologies [27].

### **1.2.2. Classifications**

CRC can be classified according to various features that influence prognosis and may predict treatment response. This section outlines selected clinically relevant classifications that informed this work, though it is not exhaustive (Figure 1.2.1).

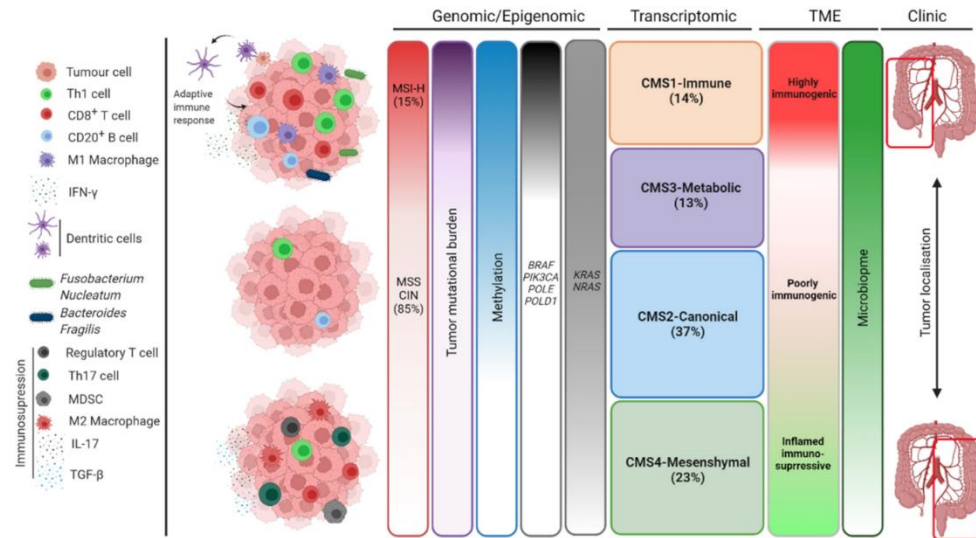


Figure 1.2.1: Colorectal cancer classifications. CIN: Chromosomal Instability, CMS: Consensus Molecular Subtype, IFN: Interferon, IL: Interleukin, MDSC: Myeloid-Derived Suppressor Cell, MSI-H: Microsatellite Instability, MSS: Microsatellite Stable, TGF: Transforming Growth Factor, Th: T-helper lymphocytes, TME: Tumour Microenvironment. Figure from Huyghe et al. [28].

### 1.2.2.1. Anatomic origin

The distinction between right- and left-sided CRCs, demarcated by the splenic flexure, stems from their embryological origin: the right colon arises from the midgut and is supplied by the superior mesenteric vessels, whereas the left colon originates from the hindgut and is perfused by the inferior mesenteric vessels [29]. Right-sided CRCs are more common in older patients and women, and are more frequently associated with mucinous histology, poor differentiation, mismatch repair deficient/high microsatellite instability (dMMR/MSI-H), and *BRAF* mutations [30-32]. In contrast, left-

sided CRCs more often harbour *KRAS* mutations and exhibit conventional chromosomal instability phenotype, whereas the CpG island methylator phenotype is more frequent in right-sided tumours [30, 31]. Prognostically, left-sided CRCs are associated with higher recurrence-free and overall survival compared to right-sided tumours [32].

#### *1.2.2.2. Disease extent*

The 8th edition of the Tumor-Node-Metastasis classification remains the standard system for staging CRC, describing disease extent and providing prognostic information (Table 1.2.1) [33].



| <b>Primary tumour (T)</b>       |                                                                                                                                                    |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| T0                              | No evidence of primary tumour                                                                                                                      |
| Tis                             | Carcinoma in situ: intraepithelial or intramucosal carcinoma                                                                                       |
| T1                              | Tumour invades submucosa                                                                                                                           |
| T2                              | Tumour invades muscularis propria                                                                                                                  |
| T3                              | Tumour invades through the muscularis propria into the pericorectal tissues                                                                        |
| T4                              | Tumour invades the visceral peritoneum or invades or adheres to adjacent organ or structure                                                        |
| <i>T4a</i>                      | Tumour invades through the visceral peritoneum                                                                                                     |
| <i>T4b</i>                      | Tumour directly invades or is adherent to other organs or structures                                                                               |
| <b>Regional lymph nodes (N)</b> |                                                                                                                                                    |
| N0                              | No regional lymph node metastasis                                                                                                                  |
| N1                              | Metastasis in 1-3 regional lymph nodes or any number of tumour deposits with negative nodes                                                        |
| <i>N1a</i>                      | Metastasis in 1 regional lymph node                                                                                                                |
| <i>N1b</i>                      | Metastasis in 2-3 regional lymph nodes                                                                                                             |
| <i>N1c</i>                      | Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized, pericolic, or perirectal/mesorectal tissues without regional nodal metastasis |
| N2                              | Metastasis in 4 or more lymph nodes                                                                                                                |
| <i>N2a</i>                      | Metastasis in 4-6 regional lymph nodes                                                                                                             |
| <i>N2b</i>                      | Metastasis in 7 or more regional lymph nodes                                                                                                       |
| <b>Distant metastasis (M)</b>   |                                                                                                                                                    |
| M0                              | No distant metastasis.                                                                                                                             |
| M1                              | Metastasis to one or more distant sites or organs or peritoneal metastasis                                                                         |
| <i>M1a</i>                      | Metastasis confined to 1 organ or site is identified without peritoneal metastasis                                                                 |
| <i>M1b</i>                      | Metastasis to two or more sites or organs is identified without peritoneal metastasis                                                              |
| <i>M1c</i>                      | Metastasis to the peritoneal surface alone or with other site or organ metastases                                                                  |

*Table 1.2.1: Tumor-Node-Metastasis (TNM) classification of the colorectal cancer.*

Based on Tumor-Node-Metastasis (TNM) categories, the American Joint Committee on Cancer defines distinct disease stages (Table 1.2.2), which guide treatment strategies.

| American Joint Committee on Cancer Stage | Corresponding Tumor-Node-Metastasis             |
|------------------------------------------|-------------------------------------------------|
| 0                                        | Tis, N0, M0                                     |
| I                                        | T1/2, N0, M0                                    |
| IIA                                      | T3, N0, M0                                      |
| IIB                                      | T4a, N0, M0                                     |
| IIC                                      | T4b, N0, M0                                     |
| IIIA                                     | T1/2, N1, M0 or T1, N2a, M0                     |
| IIIB                                     | T3/4a, N1, M0 or T2/3, N2a, M0 or T1/2, N2b, M0 |
| IIIC                                     | T4a, N2a, M0 or T3/4a, N2b, M0 or T4b, N1/2, M0 |
| IVA                                      | Any T, Any N, M1a                               |
| IVB                                      | Any T, Any N, M1b                               |
| IVC                                      | Any T, Any N, M1c                               |

*Table 1.2.2: American Joint Committee on Cancer stage for colorectal cancer.*

### 1.2.2.3. Histology

The 2019 World Health organisation classification defines four histological subtypes of CRCs. This work focuses on adenocarcinoma exclusively, which accounts > 90% of cases, while neuroendocrine tumour, neuroendocrine carcinoma, and mixed neuroendocrine-nonneuroendocrine neoplasm are not addressed here [34, 35]. Beyond the classical and highly predominant

“Adenocarcinoma not otherwise specified”, nine variants are described: adenoma-like, micropapillary, mucinous, medullary, poorly cohesive, signet ring cell, adenosquamous, sarcomatoid, and undifferentiated carcinoma [34, 35]. Among them, mucinous and signet ring cell variants are associated with poorer prognosis compared to adenocarcinoma not otherwise specified, whereas medullary carcinomas, typically dMMR/MSI-H, tend to have a more favourable outcome [35, 36].

#### 1.2.2.4. *Molecular*

Biomolecular characterisation of CRC has revealed several genetic and molecular pathways that significantly impact prognosis and response to treatment.

Overexpression of the epidermal growth factor receptor, present in 70% of CRCs, was the first identified molecular alteration [37]. Epidermal growth factor receptor activates the RAS/RAF/MEK/MAPK and PI3K/AKT/mTOR pathways, which regulate key processes in carcinogenesis such as cell proliferation, invasion, survival, angiogenesis, and metastasis [37, 38]. Epidermal growth factor receptor-targeted therapies have since been developed, but mutations in downstream effectors impact their efficacy [37, 39]. Activating *KRAS* mutation, found in approximately 40% of CRCs, confer resistance to epidermal growth factor receptor inhibitors, particularly mutations at codon 12 (G12V), which are linked to worse failure-free survival and overall survival [37-42]. *NRAS* and *HRAS* mutations are less frequent (5-10% and 1%, respectively) but also predict epidermal growth factor

receptor-inhibitor resistance [42, 43]. The activating *BRAFV600E* mutation occurs in 10% of CRCs, mainly in right-sided tumours, and is associated with poor prognosis and epidermal growth factor receptor-inhibitor resistance. *BRAF* inhibitors may be beneficial in such cases [37-39, 42]. Alterations in the PI3K/AKT/mTOR pathway also impact epidermal growth factor receptor-targeted therapy: *PIK3CA* mutations (20 – 40%) and *PTEN* loss-of-function mutations (15%) reduce treatment response [37, 38, 42].

Additionally, 5% of CRCs exhibit *ERBB2*/HER2 amplification, activating the same pathways [42]. For these patients, HER2 inhibitors may be considered in third-line metastatic settings [39].

Approximately 15% of CRCs are dMMR/MSI-H (10 – 20% in non-metastatic and 3 – 5% in metastatic CRCs) resulting from mutations in MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*) [44, 45]. dMMR/MSI-H tumours accumulate mutations in microsatellites within cancer-related genes and generate neoantigens, triggering strong immune infiltration, particularly by T lymphocytes, and responsiveness to immune checkpoint inhibitors [44-46]. Despite lower sensibility to chemotherapy, dMMR/MSI-H tumours have a better prognosis than MSS CRCs [45]. Mutations in *POLE* or *POLD1*, encoding DNA polymerase subunits, are rare (<1%) but lead to a hypermutated phenotype, associated with high immune infiltration and sensitivity to immune checkpoint inhibitors [47].

Fusion of tropomyosin receptor kinases (*NTRK*) is present in <1% of CRCs but clinically actionable, conferring sensitivity to TRK inhibitors [48].

Finally, other pathways frequently altered in CRC include Wnt (activated in 93%), transforming growth factor  $\beta$  (TGF- $\beta$ , downregulated in 36%), and TP53 (downregulated in 62%). While these contribute to tumour progression, they currently lack direct therapeutic recommendations [39, 42].

The Consensus Molecular Subtypes classification integrates gene expression profiles to define four distinct CRC subtypes [49].

- Consensus Molecular Subtype 1 (14%) – dMMR/MSI Immune: hypermutated, dMMR/MSI-H tumours with strong immune activation.
- Consensus Molecular Subtype 2 (37%) – Canonical: epithelial phenotype, chromosomal instability-driven, with activation of Wnt and MYC pathways.
- Consensus Molecular Subtype 3 (13%) – Metabolic: epithelial phenotype with marked metabolic dysregulation.
- Consensus Molecular Subtype 4 (23%) – Mesenchymal: characterised by TGF- $\beta$  activation, stromal infiltration, and angiogenesis.

This classification correlates with histological features (e.g., immune infiltration, tumour budding) and clinical outcomes, including response to chemotherapy, overall survival, recurrence-free survival, and post-relapse survival [49-52].

### **1.2.3. Tumour microenvironment**

#### *1.2.3.1. Overview*

The tumour environment is a complex ecosystem involving various elements, including immune cells, cancer-associated fibroblasts, microvasculature, extracellular matrix, signalling molecules, and the microbiota [53, 54]. This work focuses specifically on the immune compartment, and other components are not addressed here.

The tumour immune microenvironment involves a complex network of immune cells and molecular signals, encompassing a continuum from immunosuppression (pro-tumoral) to immune activation (anti-tumoral) [55].

Tumour-infiltrating lymphocytes, particularly CD8<sup>+</sup> cytotoxic T cells, play a central role in adaptive anti-tumour immunity. Upon priming by antigen-presenting cells in TDLNs, CD8<sup>+</sup> T cells can recognise tumour antigens via their T-cell receptor and induce tumour cell death through release of cytotoxic granules (granzymes, perforin, cathepsin C, granulysin) [56]. CD8<sup>+</sup> T cells exist along a spectrum ranging from naïve to activated to exhausted states [57]. A fundamental classification of T-cell subsets relies on the expression of CCR7 and CD45RA. Naïve T cells (CCR7<sup>+</sup>CD45RA<sup>+</sup>) have not yet encountered antigen; they possess high proliferative and differentiation capacity but lack immediate cytotoxic function. They constitute a reservoir that can be rapidly mobilised upon antigen exposure. Following activation and effector activity, a proportion of effector T cells persists and differentiates into memory subsets. Central memory T cells (CCR7<sup>+</sup>CD45RA<sup>-</sup>)

reside primarily in secondary lymphoid organs, exhibit limited effector function, but have a strong proliferative capacity and can readily differentiate into effector cells after antigenic restimulation. Effector memory T cells (CCR7<sup>-</sup>CD45RA<sup>-</sup>), found mainly in peripheral tissues, display reduced proliferative potential but are capable of rapid effector responses and migration to inflamed sites. Terminally differentiated effector memory cells RA<sup>+</sup> (TEMRA; CCR7<sup>-</sup>CD45RA<sup>+</sup>) emerge later in the immune response and are characterised by minimal proliferative ability but maximal cytotoxic potential [58]. In antitumor and antimicrobial responses, cytotoxic T cells also express activation markers such as CD69, CD38, and CD137 (4-1BB), reflecting active cytolytic function. Conversely, exhausted T cells upregulate inhibitory immune checkpoints after prolonged antigenic stimulation, a negative feedback mechanism that limits autoimmunity but reduces cytotoxic capacity and facilitates tumour immune escape [56, 57]. They include programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte-activation gene 3 (LAG-3), or CD39 [56, 57].

CD4<sup>+</sup> helper T cells also require priming by antigen-presenting cells through recognition of peptide-major histocompatibility complex (MHC) class II via their T-cell receptor. Upon activation, they differentiate into multiple functional subsets depending on the cytokine milieu, enabling a wide range of immune functions. Th1 cells, induced by interleukin-12 and interferon (IFN)- $\gamma$ , sustain antitumour immunity by producing IFN- $\gamma$ , interleukin-2 and tumour necrosis factor (TNF)- $\alpha$ . These cytokines promote CD8<sup>+</sup> T-cell

expansion, migration and cytotoxicity, activate natural killer cells, and stimulate myeloid populations involved in tumour destruction. Th2 cells secrete interleukin-4, interleukin-5 and interleukin-13 and are generally associated with an immunosuppressive profile through inhibition of CD8<sup>+</sup> T cells and activation of CD11b<sup>+</sup> macrophages producing TGF- $\beta$ . Nonetheless, Th2 cells may exert antitumour effects by recruiting eosinophils and alternatively activated macrophages that have with cytotoxic functions. Th17 cells, characterised by interleukin-17 and interleukin-22 production, play a dual role: they can enhance antitumour responses by recruiting dendritic cells and promoting CD8<sup>+</sup> T-cell activation, but may also support tumour progression through angiogenesis and facilitation of metastatic spread [59, 60]. As with CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells can be subdivided into naïve, central memory, effector memory and terminally differentiated effector memory RA<sup>+</sup> (TEMRA) populations based on CCR7 and CD45RA expression, which reflect their proliferative capacity and effector potential [58]. terminally differentiated effector memory RA<sup>+</sup> (TEMRA) CD4<sup>+</sup> T cells remain less clearly defined and constitute a heterogeneous population, some subsets displaying direct cytotoxic activity [61]. Regulatory T cells (Tregs) represent another CD4<sup>+</sup> subset of major importance due to their central role in tumour immune evasion. They exert various immunosuppressive effects through secretion of interleukin-10, interleukin-35 and TGF- $\beta$ , induction of apoptosis in T cells and natural killer cells, depletion of antitumour interleukin-2, and inhibition of dendritic-cell maturation and function [62].



Though less commonly targeted by treatment, B cells and plasma cells contribute to tumour immune microenvironment through cytokine production, tumour immune microenvironment organisation, antigen presentation, T-cell priming, and direct antibody-mediated effects. Their detection in the tumour immune microenvironment is associated with favourable prognosis and improved response to anti-PD-1 or anti-CTLA-4 therapies [63].

Natural killer cells, part of the innate immune system, exert direct antitumour cytotoxicity without requiring prior priming, unlike CD8<sup>+</sup> T cells. They recognise target cells via innate immune receptors and also contribute to an anti-tumour tumour immune microenvironment through cytokine secretion (e.g. IFN- $\gamma$  and tumour necrosis factor- $\alpha$ , TNF- $\alpha$ ) [64]. Natural killer T cells, sharing cytotoxic properties with natural killer cells, are associated with favourable prognosis but differ by their phenotype and the expression of a T-cell receptor that recognises lipid antigens [65, 66].

Tumour-associated macrophages are abundant in the tumour immune microenvironment and can polarise into two main phenotypes:

- M1 tumour-associated macrophages, induced by IFN- $\gamma$ , are pro-inflammatory and cytotoxic (direct or antibody mediated) via nitric oxide synthase and reactive oxygen species production.
- M2 tumour-associated macrophages, in contrast, promote immunosuppression by secreting TGF- $\beta$  and interleukin-10, and are linked to poor prognosis [67].

This polarisation is dynamic and can shift over time depending on tumour evolution [67].

Dendritic cells orchestrate T-cell priming by processing neoantigens and presenting them with co-stimulatory signals, following activation by cytokines and damage-associated molecular patterns [68]. There are three main subsets:

- Conventional dendritic cells, with strong antigen-presenting and T-cell activating capacity.
- Plasmacytoid dendritic cells, producers of type I IFNs, but with limited T-cell activation potential.
- Monocyte-derived dendritic cells, generated during chronic inflammation and often immunosuppressive, promoting Th2/Th17 over Th1 differentiation [68, 69].

Conventional dendritic cells infiltration is associated with favourable prognosis, while plasmacytoid-derived dendritic cells are linked to worse outcomes in CRC [68, 69].

Myeloid-derived suppressor cells are immature myeloid cells with major immunosuppressive functions. They inhibit T-cell proliferation, function, and migration, induce T-cell apoptosis, and secrete TGF- $\beta$  and interleukin-10, favouring an immunosuppressive microenvironment. Myeloid-derived suppressor cells also promote Th2 and Treg activation, neoangiogenesis, and tumour growth [70]. Two subtypes are distinguished by morphology and function: polymorphonuclear and monocytic [71].

Neutrophils, although abundant in blood circulation, infiltrate the tumour immune microenvironment to a lesser extent and display dual roles. Historically linked to poor prognosis due to genotoxic effects, tumour promotion, neoangiogenesis, and inhibition of natural killer and T cells, they may also contribute to anti-tumour immunity through direct cytotoxicity and enhancement of tumour-associated macrophage function in an IFN- $\gamma$ -enriched environment [72].

Interaction between immune cells in the tumour immune microenvironment can also favour the organisation into tertiary lymphoid structures, resembling lymph nodes, which are typically associated with favourable prognosis [55, 73].

Understanding this tumour immune microenvironment composition and dynamics is a key challenge to improve immune checkpoint inhibitor efficacy, especially in poorly responsive tumours such as MSS CRCs [55, 74].

#### *1.2.3.2. Tumour immune microenvironment heterogeneity*

The tumour immune microenvironment is often heterogeneous within a patient, varying across tumour regions, between primary and metastatic sites, and depending on the organ involved [25, 75]. For instance, liver metastases tend to exhibit a more immunosuppressive tumour immune microenvironment and a poorer response to immune checkpoint inhibitors compared to other sites (see Appendix 1) [25, 76].

### 1.2.3.3. *Tumour immune microenvironment in CRC*

Compared with other cancers, most CRCs fall into a tumour immune microenvironment category characterized by high angiogenic gene expression, a balanced macrophage-to-lymphocyte ratio, and a Th2 CD4<sup>+</sup> cell signature. Overall leukocyte infiltration is intermediate, lower than in melanoma, renal cell carcinoma, head and neck squamous cell carcinoma, and lung cancer (considered immune checkpoint inhibitor-sensitive), but higher than in prostate adenocarcinoma [77]. A similar difference is observed for tumour-infiltrating lymphocyte infiltration, neoantigen load, and mutational burden, all of which are higher in immune checkpoint inhibitor-sensitive tumours than in CRC [78].

tumour immune microenvironment composition in CRC varies across patients and has prognostic relevance. CRCs with strong immune infiltration, particularly CD8<sup>+</sup> cytotoxic and CD45RO<sup>+</sup> memory tumour-infiltrating lymphocytes, are associated with improved outcomes [46, 79-82]. In primary CRCs, the immunoscore quantifies CD3<sup>+</sup> and CD8<sup>+</sup> tumour-infiltrating lymphocytes density in both the tumour core and the invasive margin to assess immune infiltration on immunohistochemically stained, formalin-fixed sections. These density values are translated into percentile ranks and combined to produce a three-tier immunoscore (low, intermediate, and high) that reflects the local anti-tumour immune response. In an international validation study, the immunoscore correlates with time to recurrence, disease-free survival, and overall survival. It outperforms traditional clinical and histological parameters, including Tumor-Node-

Metastasis (TNM) staging [83, 84]. Moreover, high T-cell infiltration and a high immunoscore in metastatic sites are associated with improved disease-free survival, overall survival, and reduced metastatic burden [75].

The Consensus Molecular Subtype classes defined above are also different based on their tumour immune microenvironment composition. Compared to normal colon tissue, all Consensus Molecular Subtype classes are enriched in Tregs, except for Consensus Molecular Subtype 1, corresponding to dMMR/MSI-H tumours. These are also all enriched in memory activated CD4<sup>+</sup> tumour-infiltrating lymphocytes except the metabolic Consensus Molecular Subtype 3. Depletion was also observed for CD8<sup>+</sup> tumour-infiltrating lymphocytes, except in Consensus Molecular Subtype 1; for resting memory CD4<sup>+</sup> tumour-infiltrating lymphocytes, except in Consensus Molecular Subtype 3; and for M2 macrophages, except in Consensus Molecular Subtype 4 (mesenchymal) [51].

tumour immune microenvironment characteristics differ markedly between MSS and dMMR/MSI-H CRC. Compared with MSS, dMMR/MSI-H tumours are enriched in multiple tumour-infiltrating lymphocyte subsets, including CD4<sup>+</sup>, CD8<sup>+</sup>, and Tregs. They also express more genes linked to Th1 polarisation (*IFNG*, *TBX21*), cytotoxicity (*CD8A*), and immune checkpoints (*CTLA4*, *PDCD1*, and *LAG3*), while Th2 and Th17 associated genes are similar in both types of tumours [46, 82]. dMMR/MSI-H tumours also exhibit a significantly higher tumour mutational burden and neoantigen load than MSS cancers [46]. This “hot” tumour immune microenvironment in dMMR/MSI-H CRC helps explain their greater sensitivity and superior

outcomes with immunotherapy compared to the “cold” tumour immune microenvironment in MSS tumours [46, 81, 82].

### **1.3. Management of colorectal cancer**

#### **1.3.1. Screening**

Screening for CRC in average-risk individuals is recommended and cost-effective between ages 50 and 74 [85, 86]. It relies on faecal occult blood or faecal immunochemical tests, performed every 24 months, followed by colonoscopy if positive [86]. In Belgium, this test is free of charge, with automatic invitations sent to the eligible population [87]. Screening colonoscopy every 5 years also reduces CRC mortality compared to no screening [88]. Although multi-target stool DNA tests and computed tomography (CT) colonography demonstrate high sensitivity and specificity, they are not yet included in standard programs [88]. Given the increasing CRC incidence in younger adults and rising life expectancy, extending the screening window from age 45 to 85 in selected individuals is under consideration [4, 89]. For high-risk populations, including individuals with a history of adenomas, inflammatory bowel disease, uretero-sigmoidostomy, acromegaly, genetic predispositions (e.g. 5-fluorouracile and oxaliplatin, familial adenomatous polyposis), or family history of CRC, colonoscopy is the preferred screening method. In these cases, screening initiation age and frequency should be individually tailored [90].

### **1.3.2. Clinical presentation**

CRC may be diagnosed following the onset of symptoms, although these manifestations are often non-specific [91, 92]:

- Abdominal pain (30 – 45%)
- Rectal bleeding (20 – 40%)
- Diarrhoea (10 – 40%)
- Constipation (15 – 25%)
- Weight loss or anorexia (10 – 30%)
- Abdominal distension or bloating (10 – 15%)
- Fatigue (10%)

Clinical signs such as a palpable rectal mass or abdominal tenderness may also be present. Additionally, anaemia (haemoglobin < 12 g/dL) is observed in 20 – 25% of cases [92].

Symptoms often vary depending on tumour location. Proximal colon cancers are more likely to cause anorexia, nausea, vomiting, abdominal pain, fatigue, and anaemia, whereas distal and rectal cancers frequently present with rectal bleeding, diarrhoea, constipation, mucous stools, rectal pain, and tenesmus [93]. Importantly, a substantial proportion of patients are asymptomatic at diagnosis [91].

### **1.3.3. Diagnostic work-up**

Initial work-up is crucial as it enables accurate staging, informs treatment strategy, and allows assessment of therapeutic response. A thorough

medical history and physical examination, including direct rectal examination, should be performed to evaluate the patient's clinical condition. Blood analysis should include a complete blood count, coagulation profile, albumin, and liver and kidney function tests. Carcinoembryonic antigen quantification is also recommended for therapeutic monitoring. Dihydropyrimidine dehydrogenase mutation status should be determined to evaluate patient eligibility for fluoropyrimidine-based systemic therapies. A complete colonoscopy is required to obtain biopsy samples for histological confirmation of colorectal cancer and to assess the biomolecular profile that guide treatment decisions. Colonoscopy is also essential for detecting potential synchronous tumours. To evaluate distant disease extension, CT of the pelvis, abdomen, and thorax should be performed. Magnetic resonance imaging (MRI) is useful for characterizing indeterminate liver lesions and assessing tumour relationships with adjacent anatomical structures. Positron emission tomography-CT (PET-CT) is not part of the standard initial imaging work-up [7, 39, 94]. In rectal cancer, pelvic MRI and endorectal ultrasound are important to evaluate local tumour extension [7, 39]. Among molecular markers, only mismatch repair status is recommended in non-metastatic disease [7, 94]. In metastatic cases, biopsy analysis should include mismatch repair status, *KRAS*, *NRAS*, *BRAF*, *HER2*, and *NTRK* [39].



#### **1.3.4. Localised rectal cancer**

*(Localised rectal cancer, corresponding to American Joint Committee on Cancer stage I disease (Table 1.2.2), will not be extensively discussed, as the present work focuses on locally advanced rectal cancer (LARC) and metastatic CRC.)*

For tumours located in the upper third of the rectum, surgical resection is recommended, which may involve total mesorectal excision (TME), partial mesorectal excision, or local excision. For tumours in the mid or lower third, TME is the standard approach, except for cT1N0 tumours, where local excision remains a valid option [94, 95]. Recently, non-operative management with active surveillance has also been proposed for these low-mid tumours with the goal of organ preservation [94-96]. In patients who have undergone surgery, adjuvant chemotherapy may be considered based on risk stratification, and adjuvant chemoradiotherapy is recommended in cases of positive circumferential resection margins, pT4b, pN2 with extracapsular spread near the mesorectal fascia, or poor-quality TME [94, 95]. For dMMR/MSI-H tumours, immunotherapy followed by active surveillance without surgery is another emerging option [94, 95].

#### **1.3.5. Locally advanced rectal cancer**

LARC includes American Joint Committee on Cancer stage II and III tumours of the rectum, encompassing cT3, cT4, and/or cN+ tumours (Table 1.2.2). The treatment of these patients depends on the Tumor-Node-Metastasis (TNM) stage, the location of the primary tumour (lower-mid versus upper

rectum), the chosen strategy (surgery versus non-operative management), the MSS/MSI-H status, and the patient's condition and preferences [94, 95]. In these patients, the primary objective is to obtain local disease control [97]. This can be achieved through surgery. Among the different surgical approaches, TME provides the best local control rates, with reported two-year pelvic recurrence rates of 0 – 5% compared with 15 – 45% after local tumour resection [97, 98]. To further improve local control, pelvic RT has been evaluated [98, 99]. In a Dutch trial, 2001 patients with LARC were randomized between TME alone or TME combined with neoadjuvant short-course RT (five daily fractions). Patients treated with TME alone had a two-year local recurrence rate of 8.4%, which was significantly higher than in the RT group (2.4%,  $p < 0.001$ ) [98]. Similarly, in the Swedish Rectal Cancer Trial, patients receiving preoperative short-course RT had a five-year local recurrence rate of 11.4% compared with 26.9% in the surgery-alone group ( $p < 0.001$ ). Moreover, an overall survival improvement was demonstrated in the RT group compared with surgery alone (58% versus 48%,  $p = 0.004$ ) [99]. With regard to the timing of RT administration, local control is superior when RT is delivered preoperatively rather than postoperatively [100, 101]. In the CAO/ARO/AIO-94 trial, neoadjuvant long-course concomitant chemoradiotherapy (long-course chemoradiotherapy, 25 fractions over 5 weeks) achieved a five-year local recurrence rate of 6%, whereas postoperative RT resulted in a local recurrence rate of 13% ( $p = 0.006$ ). However, no significant difference in overall survival was observed between neoadjuvant and adjuvant long-course chemoradiotherapy ( $p = 0.80$ ) [100]. In the NSABP R-03 trial, 23.9% of patients in the preoperative long-course

chemoradiotherapy group and 27.5% in the postoperative long-course chemoradiotherapy group experienced any type of recurrence ( $p = 0.012$ ), while locoregional recurrence alone showed no difference between groups (five-year local recurrence rate of 10.7% in each group,  $p = 0.693$ ), nor did overall survival (74.5% in the neoadjuvant versus 65.6% in the postoperative RT group,  $p = 0.065$ ) [101].

Two main RT regimens are used: short-course RT delivered in five fractions over one week, and long-course RT delivered in 25 fractions over five weeks [94, 98-104]. When combined with long-course RT, the addition of a fluoropyrimidine has been shown to reduce local recurrence rate (8.1 – 8.7% compared with 16.5 – 17.1% without fluoropyrimidine,  $p < 0.05$ ) but without improving overall survival, while increasing toxicity [105, 106]. The addition of oxaliplatin as part of a doublet regimen has not demonstrated improved local control or increased pathological complete response compared to a fluoropyrimidine alone, but has been associated with a significant rise in grade  $\geq 3$  adverse events [107-109]. A delay of 4 to 12 weeks between short-course RT and surgery has been shown to improve the pathological complete response rate (10.7 – 11.7% versus 0.4 – 1.7% with immediate surgery,  $p < 0.05$ ) [110, 111]. A similar benefit of delayed surgery has been reported following long-course chemoradiotherapy [112]. In selected cases, such as cT2 N+, cT3 N0, or cT3 N+ tumours without high-risk features, surgical resection alone or combined with neoadjuvant chemotherapy (capecitabine and oxaliplatin or 5-fluorouracil and oxaliplatin) can be considered. This strategy has been shown to be non-inferior to long-course chemoradiotherapy in terms of disease-free survival and overall survival,

although its equivalence in terms of local recurrence rate has not been clearly demonstrated in patients without high-risk characteristics (cT4, cN2, involved or threatened mesorectal fascia, extramural venous invasion, or lateral lymph node involvement) [94, 95, 113, 114].

To reduce disease dissemination to distant organs and thus improve metastasis-free survival, the addition of neoadjuvant chemotherapy to either short-course RT or long-course chemoradiotherapy has been investigated in an approach known as total neoadjuvant therapy (TNT) [102-104, 115, 116]. In the phase III RAPIDO trial, TNT consisted of short-course RT to the pelvis (5 x 5 Gy), followed by 9 cycles of 5-fluorouracil and oxaliplatin or 6 cycles of Capecitabine and oxaliplatin, and then TME after 2 – 4 weeks. This strategy significantly reduced the 5-year incidence of distant metastases (23.0% versus 30.4% with long-course chemoradiotherapy alone;  $p = 0.011$ ). However, long-term results revealed reduced local control, with a 5-year local recurrence rate of 10.2% for TNT versus 6.1% for long-course chemoradiotherapy ( $p = 0.027$ ). Overall survival was not significantly different (81.7% versus 80.2%;  $p = 0.50$ ) [116]. The phase III PRODIGE-23 trial evaluated TNT with 6 induction cycles of 5-fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) followed by long-course chemoradiotherapy and TME [104, 115]. Compared with long-course chemoradiotherapy alone, TNT improved metastasis-free survival (79.2% versus 72.3%;  $p = 0.021$ ) and overall survival (81.9% versus 76.1%;  $p = 0.033$ ) without increasing local recurrence rate at 7-year follow-up [104]. The pathological complete response rate was also higher with TNT (28% versus 12%;  $p < 0.001$ ) [115].

Non-operative management has been investigated more recently to enable organ preservation [94, 103, 117]. TME is an invasive procedure that can negatively affect patients' quality of life, with faecal incontinence in around 60% of cases, major low anterior resection syndrome in almost 50%, and potential urinary and erectile dysfunction [118-120]. Achieving a clinical complete response with TNT can allow omission of surgery through close surveillance, known as the "Watch and Wait" strategy. In the phase II OPRA trial, TNT with Capecitabine and oxaliplatin neoadjuvant chemotherapy achieved a global disease-free survival of 76%, and non-operative management was offered to 74% of patients overall. In this study, induction chemotherapy was compared with consolidation chemotherapy. Patients in the consolidation arm appeared to achieve higher TME-free survival (53%) compared to induction (41%;  $p = 0.01$ ) [103]. In small tumours (cT1/2 N+ or cT3N0), organ preservation may be pursued by administering long-course chemoradiotherapy followed by an endorectal brachytherapy boost to the primary tumour [94]. The OPERA trial evaluated long-course chemoradiotherapy with either an external beam RT boost of 9 Gy in 5 fractions or an endorectal brachytherapy boost of 90 Gy in 3 fractions [109]. Patients receiving the brachytherapy boost achieved a 3-year organ preservation rate of 81% compared to 59% in the external-beam RT boost group ( $p = 0.003$ ). However, this trial excluded T4 tumours, tumours >5 cm in diameter, >10 cm from the anal verge, or involving >50% of the rectal circumference [121].

In such «Watch and Wait» protocols, restaging is performed 8 to 12 weeks after TNT and includes digital rectal examination, MRI, and endoscopy. In

cases of clinical complete response, «Watch and Wait» can be proposed with salvage surgery if local regrowth occurs. For near-clinical complete response, local excision is recommended, whereas non-clinical complete response requires TME [94].

For patients with tumours of the upper third of the rectum, neoadjuvant treatment can be omitted because of different lymphatic drainage compared with lower-mid rectal tumours, making prophylactic RT to mesorectal, internal iliac, and presacral nodes unnecessary [94, 95]. In these patients, surgery with TME or posterior mesorectal excision should be followed by adjuvant 5-fluorouracil and oxaliplatin, or chemoradiotherapy in the presence of high-risk features such as positive circumferential resection margins, pT4b, pN2 with extracapsular spread near the mesorectal fascia, or poor-quality TME [94]. As in localised rectal cancer, dMMR/MSI-H tumours can be treated with immunotherapy alone followed by active surveillance [81, 94, 95, 122, 123].

### **1.3.6. Localised and locally advanced colon cancer**

*(As for localised rectal cancer, localised and locally advanced colon cancer, corresponding to American Joint Committee on Cancer stage I-III disease (Table 1.2.2), will not be extensively discussed, as the present work focuses only on metastatic colon cancer.)*

The cornerstone of treatment for non-metastatic colon cancer is *en-bloc* colonic resection with adequate safety margins and mesenteric resection

including lymph node dissection in stage II and III disease. Adjuvant chemotherapy with the de Gramont regimen (5-fluorouracil plus leucovorin) for 6 months should be offered to patients with MSS intermediate-risk stage II disease (defined by vascular, lymphatic, or perineural invasion; histological grade 3; tumour obstruction; or preoperative carcinoembryonic antigen > 5 ng/ml). An adjuvant oxaliplatin-based doublet (5-fluorouracil/Capecitabine and oxaliplatin) administered for 6 months is recommended for patients with high-risk stage II colon cancer (pT4 disease, fewer than 12 lymph nodes examined, or multiple intermediate-risk factors) and for patients with stage III disease [7]. However, a shortened duration of 3 months of adjuvant oxaliplatin-based doublet may be considered in patients with low-risk stage III colon cancer [124].

### **1.3.7. Metastatic colorectal cancer**

Metastases are present at CRC diagnosis in approximately 22% of patients [125]. Furthermore, metastases will subsequently develop in 20 – 50% of those initially diagnosed with non-metastatic disease [39]. The 5-year overall survival for metastatic patients (14%) is markedly reduced compared with patients with localised (90%) or locally advanced disease (71%) [125]. Owing to advances such as resection in oligometastatic disease, biomolecular classification, targeted therapy, and immunotherapy, median overall survival has improved from 22.6 months (2004 – 2012) to 32.4 months (2016 – 2019) [126]. Management depends on whether the primary tumour and metastases are resectable and on biomarker status [39]. In

resectable liver disease, liver transplantation or metastasectomy provides a clear overall survival benefit with low perioperative mortality and is now standard [127, 128]. Patient selection should account for recurrence predictors, including N+ disease, a disease-free interval <12 months, multiple hepatic lesions, largest tumour >5 cm, and carcinoembryonic antigen >200 ng/ml [128]. Perioperative 5-fluorouracil and oxaliplatin does not significantly improve median overall survival (61.3 months versus 54.3 months with surgery alone,  $p = 0.34$ ) but significantly increases median progression-free survival (20.9 months with preoperative 5-fluorouracil and oxaliplatin versus 12.5 months without,  $p = 0.035$ ) [129]. When surgery is not feasible, radiofrequency or thermal ablation and stereotactic ablative RT (SABR) are valid alternatives [39, 130]. Compared with systemic therapy alone, the addition of radiofrequency ablation improves median overall survival (45.6 versus 40.5 months,  $p = 0.01$ ) and median progression-free survival (16.8 versus 9.9 months,  $p = 0.005$ ) [131]. The phase III COLLISION trial demonstrated the non-inferiority of thermal ablation in terms of local control and overall survival, with a better safety profile compared with surgical resection for CRC liver metastases [132]. This approach is limited to patients with  $\leq 3$  liver lesions, maximum lesion size  $\leq 3$  cm, no vascular invasion, and no extrahepatic disease [132, 133]. Liver SABR has shown 3-year local control of 68%, comparable to radiofrequency ablation [134, 135]. Exclusion criteria for SABR include >4 lesions, tumour diameter >6 cm, <5 mm distance from organs-at-risk (OAR), Child-Pugh score C, and a free liver volume <700 cc [136-138]. For diffuse liver metastases, intra-arterial therapies such as transarterial radioembolization, transarterial



chemoembolization, or hepatic arterial infusion may be considered [39]. These approaches are particularly relevant for liver metastases, the most common metastatic site, although local treatment of lung metastases is also feasible and has demonstrated an overall survival benefit [139, 140].

For non-resectable disease, systemic therapy should be selected based on the tumour's biomolecular profile and location [39]. In *RAS*- and *BRAF*-wild-type tumours of the left colon, first-line treatment should combine a chemotherapy doublet (e.g. 5-fluorouracil and oxaliplatin or FOLFIRI) with an anti-epidermal growth factor receptor agent in cases of epidermal growth factor receptor overexpression. In the phase III CRYSTAL study, adding cetuximab (anti-epidermal growth factor receptor) to FOLFIRI significantly improved median progression-free survival (8.0 versus 8.9 months,  $p = 0.048$ ) and median overall survival (18.6 versus 19.9 months,  $p = 0.042$ ) compared with FOLFIRI alone. This benefit is not observed in *RAS*- or *BRAF*-mutated CRC, nor in right-sided tumours [141, 142]. For these patients, a chemotherapy doublet +/- bevacizumab is preferred. The addition of bevacizumab increased median overall survival (20.3 months versus 10.6 months,  $p < 0.001$ ) compared with chemotherapy alone [143]. A chemotherapy triplet may be considered for fit patients with high-risk features and when anti-epidermal growth factor receptor therapy is less effective. Compared with FOLFIRI plus bevacizumab, adding oxaliplatin improved median overall survival (29.8 versus 48.1 months,  $p = 0.03$ ), although toxicities were significantly higher with the triplet, including more frequent grade  $\geq 3$  neurotoxicity, stomatitis, diarrhoea, and neutropenia [144, 145]. Patients with dMMR/MSI-H metastatic CRC should

receive pembrolizumab (anti-PD-1), which has demonstrated significant progression-free survival (16.5 months) and overall survival benefits (77.5 months) compared with conventional treatment (progression-free survival 8.2 months,  $p < 0.001$ ; overall survival 36.7 months,  $p < 0.05$ ) [146, 147]. A recent phase III trial investigating nivolumab plus ipilimumab (anti-CTLA-4) in first-line dMMR/MSI-H metastatic CRC showed significant progression-free survival improvement with fewer grade  $\geq 3$  adverse events compared with chemotherapy [148]. Maintenance therapy should be tailored to the first-line regimen, typically using a fluoropyrimidine with anti-epidermal growth factor receptor or bevacizumab, or irinotecan [39]. Subsequent lines of therapy depend on prior treatments and molecular alterations. Oxaliplatin- or irinotecan-based therapy should be considered depending on previous exposure [39]. In patients with a V600E *BRAF* mutation, combining anti-BRAF and anti-epidermal growth factor receptor therapy in later lines increases overall survival and overall response rate compared with chemotherapy and/or anti-epidermal growth factor receptor alone [149]. Similarly, anti-HER2 therapy is recommended from the third line in HER2-positive patients [150]. In immune checkpoint inhibitor-naïve dMMR/MSI-H patients, immune checkpoint inhibitors remain advised [39].

### **1.3.8. Immunotherapy in colorectal cancer**

As discussed previously, immune checkpoint inhibitors have demonstrated clear benefit only in dMMR/MSI-H tumours, while their efficacy in MSS tumours remains limited.

In LARC, a recent meta-analysis reported that adding immune checkpoint inhibitor to neoadjuvant management yielded a pooled pathological complete response rate of 37% and a pooled clinical complete response rate of 26% [151]. The phase Ib/II VOLTAGE trial evaluated long-course chemoradiotherapy with nivolumab in 40 MSS and 5 dMMR/MSI-H patients, showing similar clinical complete response rates (20.5% in MSS versus 20% in dMMR/MSI-H) but higher pathological complete response in dMMR/MSI-H tumours (28.9% in MSS versus 60% in dMMR/MSI-H). Three-year recurrence-free survival did not differ significantly (79.5% for MSS versus 100% for dMMR/MSI-H;  $p = 0.27$ ) [152]. In the phase II NECTAR study, adding an anti-PD-1 to long-course chemoradiotherapy followed by TME achieved a pathological complete response of 40% in 50 patients [74]. These results contrast with outcomes in dMMR/MSI-H tumours, where pathological complete response rates reached 68% with nivolumab plus ipilimumab (NICHE-2 trial) and clinical complete response rates reached 100% with dostarlimab (anti-PD-1) [81, 122, 123]. Tumour immune microenvironment analyses in dMMR/MSI-H patients treated with immune checkpoint inhibitor revealed an initial rise in CD8<sup>+</sup> and PD-L1<sup>+</sup> tumour-infiltrating lymphocytes, followed by a decrease and subsequent

resurgence, along with increased CD20<sup>+</sup> B cells and tertiary lymphoid structures [81].

In metastatic CRC, the phase II REGONIVO trial enrolled 94 previously treated MSS patients receiving regorafenib plus nivolumab. The objective response rate was 7% with a median progression-free survival of 1.8 months. Tumour immune microenvironment analysis indicated that higher baseline levels of GranzymeB<sup>+</sup> CD8<sup>+</sup> tumour-infiltrating lymphocytes, Tregs, and macrophages, as well as overexpression of inflammatory RNA signatures (T-cell activity, IFN- $\gamma$ , and expanded inflammatory signature), correlated with better outcomes. Notably, all responders were free of liver metastases [153]. Patients with liver metastases showed reduced levels of immune-sensitive cells (GranzymeB<sup>+</sup> CD8<sup>+</sup> tumour-infiltrating lymphocytes, Tregs, and macrophages), potentially explaining their poor response [154]. In the phase III IMblaze370 trial, predominantly involving MSS metastatic CRC, atezolizumab (anti-PD-1) alone or combined with cobimetinib (anti-MEK) failed to improve median overall survival (8.9 months,  $p = 0.99$  for the combination; 7.1 months,  $p = 0.34$  for atezolizumab) compared with regorafenib alone (8.5 months). The objective response rate remained  $< 5\%$  and was observed mainly in dMMR/MSI-H tumours [155]. The phase II AVETUXIRI trial assessed avelumab (anti-PD-L1), cetuximab, and irinotecan in MSS mCRC refractory to chemotherapy and anti-epidermal growth factor receptor but did not meet its primary objective response rate endpoint (10.9% overall response rate) and achieve a 6-month progression-free survival of 24.6% [156, 157]. Patients with higher baseline immunoscore (adapted for biopsies) and “distance-based immunoscore” (including

information about distance between tumour cells and T cells) had improved progression-free survival and overall survival, while memory T-cell presence correlated with progression-free survival and PD-1 expression with overall survival [157].

## **1.4. Radiotherapy**

### **1.4.1. Radiotherapy in colorectal cancer**

As described above, RT in CRC is indicated for localised and locally advanced rectal cancer as well as in metastatic CRC for a radical purpose. In metastatic CRC, SABR is an alternative option to surgery as radical treatment of liver, lung, brain, or other metastases [135, 140, 158, 159]. Since all SABR techniques vary depending on the anatomic location of the lesion and since this work focusses on liver as the only metastatic site, only liver SABR will be described here. Moreover, palliative RT (e.g. for pain or bleeding control) will not be addressed here.

#### ***1.4.1.1. Rectal radiotherapy***

In LARC, short-course RT and long-course chemoradiotherapy are the two recommended regimens. short-course RT delivers 5 fractions of 5 Gy over one week [94, 98, 99, 111, 160]. The clinical target volume (CTV) routinely includes mesorectal, presacral, and internal iliac lymph nodes. Obturator lymph nodes may be omitted in T3 tumours without extensive mesorectal lymph node involvement, and external iliac lymph nodes may be omitted in

T3 tumours without extramesorectal involvement. Inguinal lymph nodes, the ischio-rectal fossa, and the sphincter complex are included depending on pelvic organ invasion [161]. Long-course chemoradiotherapy delivers 45 – 50.4 Gy in 1.8 – 2 Gy fractions over 5 weeks, with a CTV similar to short-course RT [94, 100, 101, 103, 105-109, 112-115]. Digital rectal examination and T2-weighted MRI findings should be integrated with the planning CT to guide accurate target volume definition. In localised rectal cancer and early LARC (T1-3 N0, <40 mm), the STAR-TREC study recently showed that a non-operative strategy with long-course chemoradiotherapy (25 x 2 Gy plus capecitabine) allowed avoidance of surgery in 80% of patients at 1 year. In this study, the CTV was reduced to the mesorectum only [96].

A RT dose-response relationship in LARC supports adding a boost to the primary tumour during long-course chemoradiotherapy [162]. After long-course chemoradiotherapy, endorectal brachytherapy boosts have shown potential to increase pathological complete response rates and enable non-operative management in small tumours [121]. For external-beam RT, the benefit of a boost is debated, escalation to 54 Gy in 1.8 – 2 Gy fractions can be considered [95]. In a meta-analysis by Burbach et al., escalating the dose to 60 Gy yielded a pooled pathological complete response rate of 20.4% with acceptable acute toxicity (grade  $\geq 3$  adverse event rate of 10.3%) [163]. In the RECTAL-BOOST trial, adding 5 x 3 Gy before standard long-course chemoradiotherapy (25 x 2 Gy) did not improve pathological complete response or sustained clinical complete response rates (35.9% with boost versus 37.5% without,  $p = 0.86$ ) [164]. Additionally, patients receiving the boost reported transient declines in quality of life due to pain,

fatigue, and diarrhoea [165]. Delivering an external-beam RT boost is limited by dose constraints to OARs such as the bladder and small bowel [164]. Other uncertainties also restrict dose escalation, including rectal tumour motion and limited gross tumour volume (GTV) visualisation on cone-beam CT (CBCT) during image-guided RT, which must be accounted for in the planning target volume (PTV) margin [166, 167]. Unlike pelvic lymph node irradiation, no consensus or guideline currently exists on how to deliver a boost to the primary tumour.

#### *1.4.1.2. Liver stereotactic ablative radiotherapy*

Liver SABR is usually delivered in a small number of fractions, typically 3 to 6 on alternate days [137, 138, 168]. To achieve good local control, a biologically effective dose above 100 Gy is usually recommended, although the dose must be tailored to individual factors [137, 168]. When a tumour is near critical OARs such as the stomach or duodenum, the dose should be reduced or the number of fractions increased to respect OAR constraints [137, 169, 170]. In patients with reduced liver function, the dose should be lowered in Child-Pugh class B, and SABR is contraindicated in Child-Pugh class C [137, 169, 170].

Liver SABR faces two major challenges. The first is respiratory motion management. Liver tumour position is strongly influenced by breathing due to the close relationship between the liver and the diaphragm. This motion must be accounted for in the SABR workflow to avoid geographic miss of the target volumes [137, 169, 171]. Various strategies exist and are generally

classified as passive or active [172]. The internal target volume margin is a passive approach in which a 4D planning CT is acquired and divided into 10 phases of the respiratory cycle. The GTV is delineated on each phase and merged to generate the internal target volume, encompassing all tumour positions during breathing [172]. The mid-position strategy is another passive method, using a 4D planning CT and deformable image registration to compute the time-weighted average position of the tumour [173]. The PTV around this mid-position is defined according to van Herk's formula:

$$PTV = \alpha\Sigma + \beta\sigma - \beta\sigma_p \text{ (Eq. 1.1)}$$

where  $\Sigma$  is the standard deviations of systemic errors,  $\sigma$  the standard deviation of random errors, and  $\sigma_p$  the penumbra width. The statistical coefficients  $\alpha$  and  $\beta$  set the confidence level for meeting dose objectives. With  $\alpha = 2.5$  and  $\beta = 1.64$ , the PTV margin ensures that 90% of patients receive at least 95% of the prescribed dose [173, 174]. Abdominal compression can reduce respiratory motion and therefore liver tumour motion, allowing for smaller PTV margins [175]. Active strategies include respiratory gating, tumour tracking, and breath-hold techniques. In respiratory gating, breathing motion is monitored in real time and irradiation is delivered only during specific phases of the cycle (e.g., at the inspiratory peak) [172]. In tumour tracking, the real-time tumour position is monitored using in-room imaging and the beam is continuously adapted to follow the tumour motion [172]. In breath-hold techniques, spontaneous or induced apnoea's create a stable plateau in the inspiratory phase of the respiratory cycle, enabling irradiation with smaller PTV margins as



respiratory motion no longer needs to be encompassed [172]. Among breath-hold techniques, mechanically assisted non-invasive ventilation (MANIV) delivers positive-pressure air enriched with 60% oxygen via a facial mask, without intubation [176-179]. This approach enables stable deep-inspiration apnoea's of around 30 seconds, repeated throughout the RT session or during planning CT acquisition [176, 177]. It has been shown to be feasible and safe in liver SABR and may further reduce PTV margins compared with free-breathing techniques [178].

The second major challenge in liver SABR is the lack of visibility of the tumour on CT and CBCT images. Liver tumours generally have similar tissue contrast to surrounding liver parenchyma, making them difficult to visualise on CBCT images used for image-guided RT during treatment. To address this, surrogate markers are often used to guide the coregistration of the planning CT and CBCT during daily image-guided RT. Anatomical landmarks such as bones or diaphragmatic domes are less reliable surrogates for tumour position compared to radiopaque markers implanted percutaneously around the tumour [180, 181]. However, marker implantation is an invasive procedure that may cause pain, bleeding, or infection, and can be contraindicated in patients with liver-related comorbidities such as ascites, thrombocytopenia, or chronic liver failure [182]. An alternative approach is MRI-guided RT, which allows direct visualisation of the tumour and its motion [183]. Yet, this technique remains limited by infrastructure availability, requires significant resources and dedicated staff, takes longer than conventional RT sessions, may be contraindicated in some patients, and can provoke claustrophobia [183-187]. Due to this lack of visibility on CT,

planning MRI is also recommended to define the GTV on the planning CT [137].

#### **1.4.2. Adaptive radiotherapy**

With recent advances in computational resources, adaptive RT has gained increasing interest. The goal is to provide a more individualised treatment by accounting for anatomical changes between sessions (offline and online adaptive RT) and even within a session (real-time adaptive RT) [188]. Offline RT, where a new plan is created between sessions without the patient on the couch, and real-time adaptive RT, where the plan is adjusted while treatment is being delivered, are not discussed here [189]. For online-adaptive radiotherapy (oART), two major approaches are currently implemented in clinical practice: CBCT-guided oART and MRI-guided RT [187, 190]. As the present project focuses on developing a CBCT-guided oART workflow and comparing it with non-adaptive RT, MRI-guided RT will not be further described.

In non-adaptive RT, a planning CT is acquired before treatment, and target volumes and OARs are delineated. A treatment plan is then generated and used for all sessions. Before each session, a CBCT is acquired and rigidly registered with the planning CT during image-guided RT, improving treatment accuracy and potentially reducing toxicity [191]. The scheduled plan is then delivered without modification.

The CBCT-guided oART workflow begins similarly, with a planning CT, volume delineation, and plan generation. However, during each session, the CBCT is used for the adaptive process, performed with the patient on the treatment couch. OARs near the tumour are automatically delineated by a deep-learning segmentation algorithm, with manual corrections by a radiation oncologist if needed. Certain organs, termed “influencers,” guide a structure-based deformable image registration between the planning CT and CBCT, generating a deformation field used to propagate target volumes on the CBCT. Simultaneously, an image-based deformable registration generates a synthetic CT that accounts for electron density changes and enables creation of the remaining OARs. A daily-adapted plan is then calculated from the synthetic CT and updated contours. A comparison is made between this adapted plan and the scheduled, non-adaptive plan by the radiation oncologist based on dosimetric parameters before being delivered [190, 192, 193].

CBCT-guided oART is particularly suitable for pelvic sites, which are prone to interfraction anatomical variations, potentially opening new RT indications (see Appendix 2). Clinical workflows have already been implemented for LARC, both for short-course RT and long-course chemoradiotherapy. Reported average session durations range from 22 to 34 minutes. In both settings, adapted plans were more frequently selected than scheduled plans, resulting in significantly improved target volume coverage and better compliance with pelvic OAR constraints [194, 195].

For liver SABR, a retrospective study described 13 cases treated with abdominal compression, radiopaque markers for image-guided RT guidance, and an internal target volume approach, using CBCT-guided oART. An isotropic 3 – 5 mm PTV margin was applied. The median session duration was 29 minutes, and oART achieved the target volume coverage goal (PTV V100%  $\geq$  95%) in significantly more sessions (92%) compared to non-adaptive plans (64%;  $p = 0.042$ ). In addition, CBCT-guided oART reduced the maximum dose to OARs ( $p = 0.026$ ) [196].

## **1.5. Radiobiology and Radio-immunology**

Radiotherapy induces tumour cell death primarily through direct DNA damage and indirect injury mediated by free-radical formation, resulting in single-strand breaks, double-strand breaks, and chromosomal aberrations in tumour cells [197]. At the cellular level, this genotoxic stress activates multiple DNA repair pathways, most notably through the gatekeeper gene p53 [197, 198]. p53 activation drives the transcription of genes involved in cell-cycle arrest and DNA repair, ultimately determining cell destiny after irradiation, including apoptosis, senescence, or autophagy [199]. Irradiated cells also release exosomes enriched in p53-induced proteins that can act on neighbouring cells [200]. Proteins of the p53 signalling pathway are detectable in the bloodstream and may serve as potential biomarkers of radiation exposure [201].

Beyond direct cytotoxicity, radiotherapy elicits both local and systemic immune responses in which T cells play a central role. Irradiation promotes

tumour neoantigen release and activates the cGAS–STING pathway, inducing type I interferon production and upregulation of MHC-I on tumour cells, thereby enhancing antigen presentation. Tumour neoantigens are peptides generated by tumour-specific somatic mutations (e.g., nonsynonymous single-nucleotide variant, insertion, deletion) that are processed and presented by human leukocyte antigen (HLA/MHC) molecules on malignant cells and can be specifically recognized by T cells [202]. Because the mutated sequence is absent from normal tissues, neoantigens represent truly “non-self” antigens with limited central tolerance, making them compelling targets for cancer immunotherapy. Concurrently, damage-associated molecular patterns recruit and mature dendritic cells, which migrate to tumour-draining lymph nodes to prime naïve T cells. The resulting expansion of antitumour T-cell clones leads to their systemic dissemination and infiltration of irradiated and distant lesions, where they can mediate immunogenic tumour cell death [203-207]. Other immune populations, including Tregs, myeloid-derived suppressive cells, tumour associated macrophages, and natural killer cells as well as stromal and endothelial cells, also contribute to this complex RT-induced immune response. Its magnitude and quality depend on multiple parameters, including radiation dose, fractionation, timing, and target volume, which will be further discussed below. In MSS colorectal cancer, neoantigens are of low diversity and typically arises from punctual mutations, resulting in poor immunogenicity, limited immune infiltration, and suboptimal responses to immune checkpoint inhibitors. This contrasts with MSI-H tumours, characterised by high tumour mutational burden,

abundant immunogenic neoantigens, dense immune infiltration, and strong responses to immunotherapy [46]. In vitro, in poorly immunogenic models, radiotherapy has been shown to increase neoantigen diversity and restore sensitivity to immune checkpoint blockade above specific dose thresholds (absent at 2 Gy, present at 4 Gy) [208]. Accordingly, this project evaluates radiotherapy as a strategy to enhance immunogenicity in MSS colorectal cancer, where immune responsiveness is typically low [209].

Independent of its direct cytotoxic and immunomodulatory effects, radiotherapy also alters tumour perfusion by acting on endothelial cells and the vascular architecture. These vascular effects are complex and highly dose-dependent. Low-dose irradiation can transiently enhance tumour perfusion, whereas higher doses induce vascular damage leading to reduced perfusion and indirect tumour cell death [210]. The transition between these two states is narrow and not clearly delineated. For example, a preclinical study reported a modest, non-significant 4.3% reduction in blood flow following a single dose of 8 Gy whereas other studies demonstrated substantial vascular destruction at 10 Gy, potentially impairing immune-cell migration into the tumour microenvironment [210-212]. On the other hand, low dose radiotherapy potentially stimulates angiogenesis and improves blood vessel permeability [210, 213, 214]. Both primary colorectal tumours and their metastases typically exhibit a dense yet highly disorganised vascular architecture [215]. In liver metastases, vascular organisation further differs between histopathological growth patterns: desmoplastic lesions display chaotic neoangiogenesis; replacement lesions grow by co-opting pre-existing hepatic vessels; and pushing lesions compress surrounding liver

tissue while relying on neoangiogenesis [216, 217]. As a result, both primary and metastatic CRC lesions are marked by inefficient perfusion and a consequently hypoxic microenvironment [218, 219].

### **1.5.1. Radiotherapy dose and immune system**

Radiotherapy modify the local tumour immune microenvironment, with effects that vary according to dose (Figure 1.5.1). Across most dose levels, increased infiltration of CD8<sup>+</sup> T cells and natural killer cells has been consistently observed [220-224]. Tregs, which are relatively radioresistant, expand linearly with doses ranging from 2 to 20 Gy [225]. Notably, high-dose-induced Treg accumulation can be reversed by subsequent low-dose irradiation [226]. Tumour-associated macrophages also exhibit dose-dependent polarisation: low-dose RT (2 Gy per fraction) favours antitumoral M1 macrophages, whereas higher doses (>8 Gy per fraction) promote protumoral M2 phenotypes associated with angiogenesis and immunosuppressive cytokine secretion [67, 213, 227, 228]. Dendritic cells mediate RT-induced antitumour immunity through distinct dose-specific mechanisms: low-dose RT increases production of pro-immunogenic cytokines that support T-cell proliferation, while higher doses (>8 Gy per fraction) enhance MHC-II and co-stimulatory receptor expression required for efficient T-cell priming [221, 229, 230]. High-dose RT reduces myeloid-derived suppressor cells, while moderate- and low-dose RT increase their infiltration, contributing to local immunosuppression [205, 231, 232].

RT-induced systemic immune responses are likewise dose-dependent. Vanpouille-Box et al. demonstrated that high-dose RT (> 12 Gy per fraction) induces the exonuclease Trex1, which degrades cytosolic DNA necessary for STING-mediated interferon- $\beta$  production and CD8<sup>+</sup> T-cell activation, thereby limiting the abscopal effect compared with moderate-dose RT (8 Gy per fraction) [233]. Dewan et al. further showed that fractionation enhances systemic responses: three fractions of 8 Gy generated stronger interferon- $\beta$  induction than a single 8-Gy fraction [233, 234]. By contrast, low-dose RT (<2 Gy per fraction) does not induce sufficient tumour cell death to release neoantigens and damage-associated molecular patterns, nor does it increase CD8<sup>+</sup> T-cell infiltration in distant lesions to the same extent as higher doses (>8 Gy) [235]. Nonetheless, low-dose RT to secondary lesions can reduce their TGF- $\beta$ 1 expression, contributing to a more permissive immune environment [236].



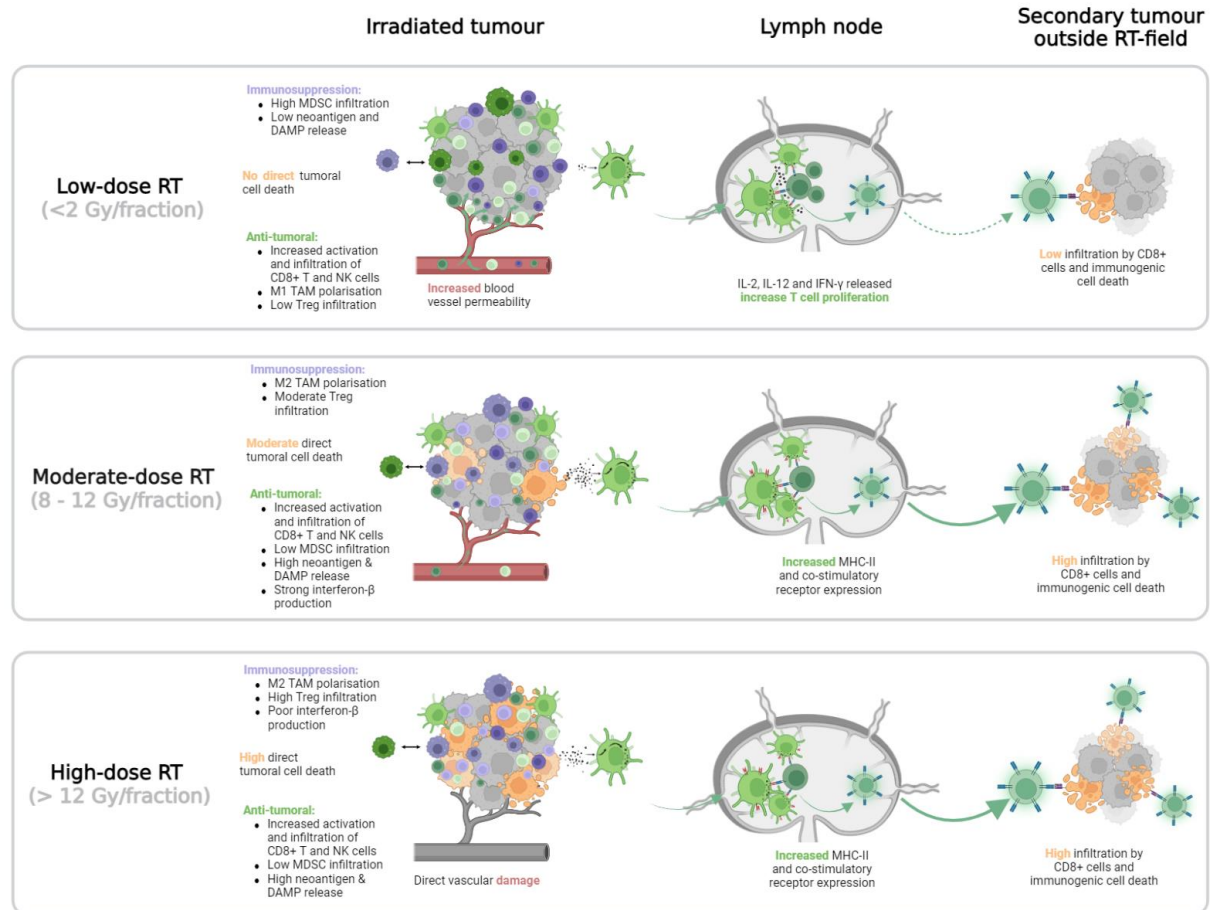


*Figure 1.5.1: Tumour immune microenvironment modifications and systemic reactions induced by different RT dose levels. Unlike moderate- or high-dose RT, low-dose RT does not induce sufficient direct tumour cell death to release enough neoantigens and damage-associated molecular patterns, resulting in a weak distant immune response. In high-dose RT, CD8+ priming and systemic activation are limited by induction of the Trex1 exonuclease. Compared with high-dose RT, low-dose RT may reduce Treg infiltration in the irradiated tumour and promote M1 polarisation of tumour-associated macrophages, thereby enhancing antitumour activity. Moderate and high-dose RT decrease Myeloid-derived suppressor cell infiltration, whereas low-dose RT increases it. dendritic cells are key to CD8+ T cell priming in tumour-draining lymph nodes. At low doses, this effect is mainly driven by dendritic cell production of interleukin-2, interleukin-12, and IFN- $\gamma$ , while at higher doses it results from increased MHC-II expression on dendritic cells. Tumour immune microenvironment remodelling is also shaped by RT effects on vasculature: low-dose RT enhances vascular permeability and immune cell migration, whereas high-dose RT impairs migration by causing direct vascular damage. DAMP: damage-associated molecular pattern, MDSC: Myeloid-derived suppressive cell, MHC: Major histocompatibility complex, NK: Natural-killer, RT: Radiotherapy, TAM: Tumour-associated macrophage, Treg: Regulator T cell. Adapted from Pierrard et al. [25].*

### **1.5.2. Radiotherapy volume and immune system**

Lymphopenia is a well-established consequence of radiotherapy and affects circulating T cells, B cells, and natural killer cells to a similar extent. This depletion can be prolonged, with recovery often taking several months [237]. Its severity correlates with poorer oncological outcomes, particularly in trials combining RT with immunotherapy [238-240]. In patients receiving RT-immunotherapy combinations, low absolute lymphocyte counts have been associated with reduced response rates in non-irradiated lesions compared with patients with higher lymphocyte levels [239]. RT-induced lymphopenia is strongly linked to the radiation dose delivered to hematopoietic organs, such as bone marrow, spleen, and heart with greater impact seen in non-conformal techniques (e.g., 3D RT) compared with intensity modulated RT, as well as with larger target volumes and higher numbers of treatment fractions [240-244]. Additional risk factors include older age, female sex, and low baseline lymphocyte counts, whereas concurrent chemotherapy appears to have minimal influence on RT-induced lymphopenia [245]. Therefore, reducing irradiated volume can preserve circulating lymphocyte population [238]. Reducing irradiated volume therefore helps preserve circulating lymphocyte populations, as demonstrated in stereotactic RT, where treatment fields and the number of fractions is markedly reduced relative to normofractionated RT [240, 246].

In rectal cancer, irradiation of TDLNs is recommended for curative treatment because it improves local control [100]. However, because antitumour immune priming occurs in TDLNs, their irradiation can be detrimental when

RT is combined with immunotherapy [247-251]. In preclinical models, TDLN irradiation (or surgical removal) reduces chemoattractant chemokine signalling, decreases CD8<sup>+</sup> T-cell infiltration and M1/M2 macrophage ratios within both irradiated and distant tumours, and impairs systemic immune responses, thereby limiting abscopal effects. These findings were consistent whether or not RT was combined with immunotherapy and irrespective of the immune checkpoint inhibitor used (anti-PD-1 or anti-CTLA-4) [247, 248, 250, 251]. In humans, the negative outcomes of major phase III trials evaluating concurrent chemoradiation and immunotherapy in head-and-neck and non-small-cell lung cancer may partly reflect extensive radiation fields that included TDLNs, thereby suppressing RT-immune checkpoint inhibitor synergy [252, 253]. A potential strategy to overcome this is to delay TDLN irradiation relative to primary tumour RT: initial irradiation of the primary lesion may prime an effective immune response and enhance immunotherapy activity while preserving TDLN structure and function, with delayed TDLN treatment introduced later [249, 254].

There is growing consensus that multi-site ablative radiotherapy, rather than single-site irradiation, should be preferred when combined with immunotherapy in the metastatic setting [255-260]. In preclinical models, irradiation of two tumour sites produced a faster and stronger response in a distant, non-irradiated lesion compared with irradiation of a single site [261]. Given the molecular heterogeneity between metastases, targeting multiple lesions increases the diversity of tumour-derived neoantigens. Their processing by dendritic cells broadens the T-cell repertoire and may reduce the stereotypical nature of the antitumour immune response [259,

262]. However, in patients with extensive metastatic burden, SABR cannot always be safely delivered to all sites without exceeding normal-tissue tolerance. In such cases, a combined strategy using moderate- to high-dose RT (6 – 12 Gy or >12 Gy per fraction) on one selected lesion and low-dose RT (total dose  $\leq 10$  Gy in fractions  $\leq 2$  Gy) on all remaining sites may enhance cytotoxic T-cell infiltration and induce regression in lesions receiving low-dose RT. This “RadScopal effect” has been demonstrated in different preclinical studies [258, 263]. Immunologically, heterogeneous tumour irradiation combines the immunostimulatory effects of low-dose RT together with the cytotoxic efficacy of high-dose RT. In preclinical colorectal and breast cancer models, partial tumour irradiation combining low-dose (2 Gy) and high-dose (16 Gy) regions resulted in superior tumour control and survival when combined with anti-PD-1 therapy, compared with uniformly delivered 2-Gy or 16-Gy regimens, or partial 16-Gy irradiation with the remaining tumour left untreated (0 Gy). This heterogeneous irradiation pattern enhanced infiltration and activation of antitumour CD8<sup>+</sup> T cells, although it also increased protumoral neutrophils, a phenomenon that could be mitigated with targeted immunotherapy to further improve outcomes [264].

### **1.5.3. Radiotherapy timing and immune response**

Understanding the temporal relationship between irradiation and the ensuing immune response is critical for optimising RT–immunotherapy combinations. However, the optimal sequencing of these modalities

remains uncertain. Accumulating evidence suggests that initiating treatment with RT followed by delayed immunotherapy may be superior to other strategies [265, 266].

In preclinical models, administration of anti-PD-1 therapy after RT has been shown to increase the number and functionality of intratumoural CD8<sup>+</sup> T cells and to enhance tumour responses outside the irradiated field. In contrast, delivering immunotherapy prior to RT results in reduced intratumoural CD8<sup>+</sup> T-cell infiltration and diminished distant tumour control [265]. Importantly, the therapeutic window for post-RT immunotherapy appears to be narrow. In a preclinical colorectal cancer model, a single 10-Gy RT fraction induced a peak in PD-1 expression on T cells approximately 5 days after irradiation, leading to CD8<sup>+</sup> T-cell exhaustion and apoptosis. Administration of anti-PD-L1 therapy at this time point counteracted the loss of CD8<sup>+</sup> T cells and achieved superior tumour control compared with earlier (1 or 3 days) or later (7 days) treatment, none of which resulted in meaningful tumour control [266].

Clinical data further support a sequential rather than concurrent approach. This is illustrated by two pivotal phase III trials in non-small cell lung cancer: PACIFIC and PACIFIC-2. In the PACIFIC trial, chemoradiotherapy followed by consolidation immunotherapy significantly improved progression-free and overall survival compared with chemoradiotherapy alone [267, 268]. In contrast, the PACIFIC-2 trial, in which immunotherapy was administered concurrently with chemoradiotherapy, failed to demonstrate a benefit for either endpoint compared to chemoradiotherapy alone [269].



## Chapter 2

# 2 Thesis objectives



## **2.1. Rationale for the project**

The rationale for this project is grounded in several well-established observations outlined in the introduction:

- CRC represents a major public health burden [1]
- The prognosis of MSS CRC remains poor [125, 126]
- Immunotherapy has demonstrated only limited efficacy in metastatic MSS CRC [153, 155, 157]
- clinical trials investigating RT-immunotherapy combinations in MSS CRC have thus far yielded modest results [270, 271]

Nevertheless, the marked efficacy of immunotherapy in dMMR/MSI-H CRC, together with preclinical evidence supporting RT-induced immune modulation and synergy with immunotherapy, provides a strong rationale for exploring RT–immunotherapy combinations as a strategy to restore immunotherapy sensitivity in MSS tumours. Conceptually, this approach aims to convert immunologically “cold” MSS tumours into a more dMMR/MSI-H–like, inflamed phenotype with an effective antitumour immune response. Before initiating clinical trials of RT–immunotherapy combinations in MSS CRC, a comprehensive understanding of the biological mechanisms underlying RT-induced immune responses in humans is an essential prerequisite to rationally design treatments that maximise antitumour immunity. Accordingly, the primary objective of this project is to characterise the immune response induced by RT in patients with MSS CRC through the analysis of irradiated tumour samples. This main project is detailed in Section 4.3 and Appendix 10 of this manuscript. To this end, a

dedicated “window-of-opportunity” protocol was implemented, in which patients receive RT prior to planned surgery for a primary or metastatic MSS CRC lesion. As discussed in previous sections, RT-induced immune responses are not uniform but rather reflect a balance between antitumour immunostimulatory and protumoural immunosuppressive signals. Consequently, substantial preliminary work was undertaken to define an optimal RT strategy that maximises the likelihood of inducing a favourable antitumour immune response. This RT regimen was established based on both biological rationale and pragmatic considerations, taking into account clinical feasibility and ethical constraints.

Different RT dose regimens were selected for evaluation. The primary regimen of interest was 3 x 8 Gy, which was delivered to all patients. This dose was chosen because it induces sufficient tumour cell death to promote neoantigen release while remaining below the threshold associated with TREX1 activation, which can limit radiation-induced out-of-field immune responses [233]. In addition, fractionated RT has been shown to elicit a stronger immune response than single-fraction irradiation [234]. A second RT regimen, 3 x 1 – 2 Gy, was evaluated in patients with multiple lesions. Low-dose RT is known to induce a distinct immune response compared with moderate-dose regimens such as 3 x 8 Gy, notably by reducing intratumoural Treg infiltration [225]. The dose range of 3 x 1 to 3 x 2 Gy was deliberately selected to ensure clinical feasibility. For comparative purposes, non-irradiated lesions (<3 x 0.5 Gy) from irradiated patients with multiple lesions, as well as lesions from patients who had never received RT, were used as controls for immune profiling. High-dose RT (>12 Gy per fraction) was not

included in this study because such doses have been shown to impair distant immune responses through TREX1-mediated mechanisms [233]. Moreover, high-dose RT induces substantial vascular damage, which may restrict immune cell trafficking into the tumour microenvironment, providing an additional rationale for avoiding these regimens [210]. Finally, as participation in this study was voluntary and not intended to confer direct clinical benefit, patient safety and preservation of standard oncological management, the subsequent surgery, were prioritised. High-dose RT was therefore excluded to minimise the risk of radiation-related toxicity and potential surgery-associated complications.

With respect to irradiated volumes, particular attention was paid to preserving the patient's immune system from unnecessary radiation exposure. Stereotactic RT was therefore employed to minimise the total blood volume exposed during the experimental procedure. In addition, strict dose constraints were applied to organs at risk involved in haematopoiesis and blood circulation (i.e. heart, spleen, and great vessels), as well as to tumour-draining lymph nodes (i.e. liver hilum and pulmonary hilum), following the "as low as reasonably achievable" (ALARA) principle [240-244, 247-251]. A pre-specified distinction was made according to the irradiated site, given the potential differences in both local and systemic tumour-immune environments. This included non-metastatic patients irradiated at the primary tumour site (reflecting an immune milieu that has not yet permitted metastatic dissemination), patients irradiated at liver metastases (a context associated with local and systemic immunotolerance and reduced immunotherapy efficacy), and patients with non-hepatic metastases, in

whom the immune environment may represent an intermediate state [25, 76]. As the objective of this study was not to achieve direct clinical benefit from irradiation, only a single lesion was irradiated at a moderate dose (3 x 8 Gy), despite evidence suggesting that multi-site irradiation may enhance immunological and clinical outcomes [255-260]. This approach also enabled the evaluation of additional dose levels within the same protocol (3 x 1 – 2 Gy and <0.5 Gy in 3 fractions) while limiting the risk of RT-related adverse events. Given the anticipated constraints on patient recruitment, eligibility was not restricted to patients presenting with at least three distinct lesions amenable to irradiation at the three planned dose levels. Consequently, all patients with MSS CRC scheduled for surgery were eligible, including those with a single lesion irradiated at 3 x 8 Gy. Accordingly, comparisons of RT-induced immune responses were performed between lesions rather than between patients.

A delay of 4 to 8 days between completion of RT and surgery was selected. From a biological perspective, this interval is expected to allow the development of measurable local and systemic immune responses, based on preclinical data [266, 272]. It also avoids the peak of RT-induced fibrosis, which typically occurs approximately two weeks after irradiation and could complicate the surgical procedure and compromise patient safety. RT has been shown to induce a biphasic activation of transforming growth factor- $\beta$  (TGF- $\beta$ ) and other cytokines, including interleukin-1, promoting fibroblast proliferation, extracellular matrix expansion, and collagen deposition. The initial peak of collagen accumulation occurs around two weeks post-RT, followed by a transient decline and a subsequent increase during the chronic

phase [273-275]. From a practical and ethical standpoint, this timing also ensured that surgery was not significantly delayed. Between the surgical decision and the operative date, the protocol requires a dedicated consultation, RT planning CT, RT delivery, and the post-RT interval of 4 – 8 days, a sequence that is difficult to compress into less than two weeks.

The development of this protocol also provided an opportunity to implement and evaluate modern RT techniques that, in some cases, enable more precise treatment delivery and, in others, reduce treatment invasiveness. For this purpose, two anatomical sites frequently irradiated in the management of CRC were specifically addressed: the rectum (Section 3.1 and Appendices 4, 5, and 6) and the liver (Sections 4.1 and 4.2 and Appendices 8 and 9). These two sites present distinct technical challenges. Rectal tumours are located in close proximity to their tumour-draining lymph nodes and exhibit unpredictable motion, whereas liver tumours display predominantly respiration-induced, more predictable motion and are not directly visualised on cone-beam computed tomography (CBCT) during conventional image-guided RT. To address these challenges, two technologies recently implemented in the RT department where this research was conducted were utilised: CBCT-guided online adaptive radiotherapy (oART) (Sections 3.1 and 4.1 and Appendices 4, 5, 6, and 8) and mechanically assisted non-invasive ventilation (Section 4.2 and Appendix 9). During the course of this project, additional research questions emerged, predominantly in the context of locally advanced rectal cancer. Although not formally recommended, focal rectal tumour irradiation is widely implemented in clinical practice to deliver an external-beam RT boost to the

primary tumour during neoadjuvant long-course RT for locally advanced rectal cancer [103, 115]. Through an extensive synthesis of the literature in the form of a systematic review, several technical parameters associated with boost delivery were identified as being correlated with clinical outcomes. This work provides a strong rationale for the development of international recommendations on the optimal delivery of rectal RT boosts (Section 3.2 and Appendix 7.7). Building on development made in rectal RT dose escalation, reductions in irradiated volumes, accumulated experience with advanced biomolecular techniques, and recent developments reported in the literature, a clinical study proposal in locally advanced rectal cancer was subsequently developed (Section 3.3). This protocol should be regarded as a perspective arising from this thesis rather than a completed project and may serve as a foundation for future discussion and refinement of its design.

Although the chronological development of the work could be described as follows:

1. Development of online adaptive workflows for rectal and liver tumours (Sections 3.1 and 4.1).
2. Conduct of translational analyses aimed at characterising the impact of RT on the tumour immune microenvironment in patients with MSS CRC (Section 4.3).
3. Further optimisation and mechanistic understanding of RT techniques (Sections 3.2 and 4.2).
4. Conceptualisation of a clinical protocol for locally advanced rectal cancer as a perspective of this thesis (Section 3.3).

The manuscript was deliberately structured to reflect the clinical evolution of CRC from non-metastatic disease (Section 3) to metastatic disease (Section 4). Within each of these two main sections, technical developments in RT are presented first (Sections 3.1, 3.2, 4.1, and 4.2), followed by translational investigations addressing the interactions between RT and the immune system (Sections 3.3 and 4.3).

## **2.2. Locally advanced rectal**

A workflow using CBCT-guided oART is investigated to assess the benefit of oART in reducing the PTV for the rectal boost during long-course chemoradiotherapy, escalating the dose to the primary tumour, and potentially increasing the tumour control probability. In this study, uncertainties and errors related to oART are quantified and incorporated into a PTV margin using the van Herk formula and compared with those of non-adaptive RT. This work has been published in *Physics and Imaging in Radiation Oncology* [276].

Second, a systematic literature review with meta-analysis evaluates clinical and technical practices for rectal boost RT. The meta-analysis aims to determine which planning parameters are associated with clinical outcomes, particularly the pathological complete response rate. This work has been published in *Clinical and Translational Radiation Oncology* [277].

Third, as a perspective in locally advanced rectal cancer, a preliminary phase II clinical study protocol is proposed and discussed to investigate a novel TNT

approach. This strategy combines dose-escalated RT to the primary rectal tumour with delayed irradiation of tumour-draining lymph nodes, together with immunotherapy and induction chemotherapy. The protocol is exploratory and intended as a foundation for future optimisation prior to potential initiation and submission. It has not been published.

### **2.3. Metastatic colorectal cancer**

First, CBCT-guided oART is investigated as a strategy for markerless liver SABR. In this *in silico* study, the accuracy of different methods for automatic target volume propagation from the planning CT to the CBCT is assessed. The resulting propagation uncertainty is quantified and incorporated into the PTV margin. A dosimetric comparison is then performed between conventional liver SABR with radiopaque markers and CBCT-guided oART without markers, integrating the propagation error into the PTV. This work has been published in *Physics in Medicine & Biology* [278].

Second, MANIV-induced BH is evaluated for liver SABR, particularly regarding CBCT image quality and its clinical impact. MANIV-BH and free-breathing CBCTs are compared in terms of image quality, coregistration accuracy with the planning CT, precision of automatic target volume propagation, and performance of deep-learning-based OAR autosegmentation. This work has been published in *Clinical and Translational Radiation Oncology* [279].



Third, a translational prospective study investigates the impact of RT on the tumour immune microenvironment, considering RT dose and site. In this window-of-opportunity study, patients scheduled for surgical resection of CRC lesions (primary or metastatic) receive preoperative irradiation. Safety and feasibility are evaluated, and blood and tumour samples are collected to characterise the immune environment through multiparametric analyses, including fluorescence-activated cell sorting, RNA sequencing, proteomics, and multiplex immunofluorescence. This work is not yet published.

## **2.4. Additional contributions**

The appendices include further contributions providing background and supporting data for this manuscript:

- Appendix 1: A literature review describing immune differences between anatomical sites commonly involved by metastases and their modulation by RT. Published in *Biomolecules* [25].
- Appendix 2: The first reported case of CBCT-guided oART for unresectable colon cancer. Published in *Technical Innovations & Patient Support in Radiation Oncology* [280].
- Appendix 3: Propensity score-matched retrospective study evaluating the impact of RT on liver allograft rejection mediated by RT-impacted immune cells, such as T cells. Published in *Cancer/Radiothérapie* [281].

- Appendix 4: A study comparing intrafraction rectal motion patterns between patients receiving RT for LARC and for prostate cancer, serving as preliminary work for CBCT-guided oART in rectal boost. Published in *Technical Innovations & Patient Support in Radiation Oncology* [282].
- Appendix 5: A study quantifying interobserver variability in rectal boost delineation involving radiation oncologists and radiotherapy technologists, also serving as preliminary work for CBCT-guided oART in rectal boost. Published in *Technical Innovations & Patient Support in Radiation Oncology* [283].



## Chapter 3

# 3. Locally advanced

# rectal cancer

### **3.1. Investigation of changes in planning target volume and regression probability of rectal boost using in-silico cone-beam computed tomography-guided online-adaptive radiotherapy**

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#### **Highlights**

- Online-adaptive radiotherapy reduces rectal boost planning target volume
- High- & low-dose target volume coverage is better with online-adaptive radiotherapy
- A 60 Gy adaptive rectal boost spares organs similarly to a non-adaptive 54 Gy boost
- Online-adaptive radiotherapy dose escalation could improve rectal tumour control

## **Abstract**

### *Background and purpose*

Radiotherapy boost to the primary tumour may enable organ preservation in locally advanced rectal cancer (LARC). This study evaluated cone-beam computed tomography (CBCT)-guided online-adaptive radiotherapy (oART) to reduce rectal boost planning target volume (PTV<sub>Boost</sub>) margins and allow dose escalation.

### *Materials and methods*

Eleven LARC patients were included in this *in silico* study. Population-based PTV<sub>Boost</sub> margins were computed for non-adaptive and oART using van Herk's formalism. Dose/volume results were compared between: non-adaptive RT with a 25 x 2.16 Gy boost (Non-ART<sub>54Gy</sub>), ART with a 25 x 2.16 Gy boost (ART<sub>54Gy</sub>), and ART with an escalated boost of 25 x 2.4 Gy (ART<sub>60Gy</sub>). Tumour regression probability was compared between each plan using a dose-response model.

### *Results*

PTV<sub>Boost</sub> margins for non-adaptive versus oART were 14.2 vs. 3.3 mm in the antero-posterior, 5.0 vs. 3.2 mm in the left-right, and 12.3 vs. 8.7 mm in the supero-inferior axes. PTV<sub>Boost</sub> and pelvic lymph node PTV coverage (V95%) were significantly improved with ART<sub>54Gy</sub> and ART<sub>60Gy</sub> compared to Non-ART<sub>54Gy</sub> ( $p < 0.001$ ). High-priority organ-at-risk constraints (priority 1&2) were violated in 26.8% of cases for Non-ART<sub>54Gy</sub>, 21.2% of cases for ART<sub>54Gy</sub>, and 20.8% of cases for ART<sub>60Gy</sub>. Tumour regression probability was superior for

ART<sub>60Gy</sub> (20.8%) compared to ART<sub>54Gy</sub> (17.0%,  $p<0.001$ ) and Non-ART<sub>54Gy</sub> (16.9%,  $p<0.001$ ).

### *Conclusions*

oART significantly reduce rectal boost PTV margin. It allows better target volume coverage with a similar risk of radiation-induced toxicities, even when escalating the dose. Therefore, oART should be considered to perform dose-escalation in LARC patients with the objective of organ preservation.

### **3.1.1. Introduction**

Rectal cancer represents about 30% of all colorectal cancer. For locally advanced rectal cancer (LARC), current management combines radiotherapy (RT), chemotherapy, and surgery [284, 285]. RT and chemotherapy can be given concomitantly during a long-course radiochemotherapy as part of total neoadjuvant therapy [103, 115, 163, 285, 286]. More recently, TNT combined with induction or consolidation chemotherapy can also be used as a potential definitive treatment under a “watch and wait” strategy for organ preservation [103, 287]. To improve clinical complete response rates, a RT boost to the primary tumour can be advised, either using external-beam RT or contact x-ray brachytherapy [103, 121, 164, 288-291]. Contact x-ray brachytherapy has shown promising organ preservation rates in early stages, but data for LARC remain scarce [121, 291]. Using external-beam RT, clinical complete response rate can reach nearly 50% [103]. However, dose escalation to the primary tumour is controversial due to potential excessive toxicities [165]. Unlike contact x-ray brachytherapy, external-beam RT must integrate the tumour interfraction motion in the boost planning target volume ( $PTV_{\text{Boost}}$ ), increasing the total irradiated volume [292-296]. This limits the opportunity for external-beam RT dose escalation, which would potentially increase the complete response rate [162, 163].

Recent technical innovations could be used for dose-escalation of the primary rectal tumour. Among them, cone-beam computed tomography (CBCT)-guided online-adaptive RT (oART) is an emerging technology enabling daily delineation of organs-at-risk (OARs) and target volumes, with



daily planning in an acceptable time for clinical application [190, 192]. This technique is already used in neoadjuvant rectal cancer treatment [194]. By eliminating the interfraction motion impact, CBCT-guided oART can potentially reduce the PTV margins and allow dose-escalation compared to non-adaptive strategies.

The first objective of this *in silico* study was to compute population-based PTV margins for rectal boost external-beam RT. We determined the PTV margin that should be applied in non-adaptive and in CBCT-guided oART strategies to assess whether oART allowed a PTV reduction. The second objective was to perform a dose-volume analysis evaluating if CBCT-guided oART allows primary tumour dose escalation while preserving pelvic OARs.

### **3.1.2. Materials and Methods**

#### **3.1.2.1. Patients**

From January to October 2023, we prospectively included 11 patients treated by RT for rectal tumour in our hospital. Children and patients with prior pelvic RT were excluded. No other inclusion or exclusion criteria applied. This study followed the Declaration of Helsinki and was approved by the ethics committee of the Cliniques universitaires Saint-Luc (reference number: B4032022000061 – July 22, 2022). All participants provided written informed consent.

### 3.1.2.2. *Treatment*

A 3-mm-slice-thickness planning-CT (Aquilion LB, Toshiba Medical Systems, Tokyo, Japan) was acquired with intravenous iodine contrast (120 kVp, 400 mAs). The planning-CT and RT sessions were performed supine, with a comfortably full bladder (300 mL water, 30 minutes prior), without rectum preparation. The RT planning was done on the RayStation Planning system (clinical versions 9B and 12A, RaySearch Laboratories, Stockholm, Sweden). The boost gross tumour volume ( $GTV_{boost}$ ) was the primary tumour delineated on the planning CT, adapted according to clinical examination, rectoscopy, and a rigid co-registration with T2-weighted magnetic resonance imaging (MRI, 3.5 mm slices, Ingenia 3T, Philips Healthcare, Amsterdam, The Netherlands or SIGNA Premier 3T, GE Healthcare, Boston, MA, USA). The clinical boost target volume ( $CTV_{boost}$ ) included the  $GTV_{boost}$  and the circumferential rectal wall at each transversal CT slice where the tumour was present. The elective CTV encompassed lymph node areas according to international recommendations [161]. The RT was delivered by two linear accelerators (Halcyon® and Ethos®, Varian a Siemens Healthineers Company, Palo Alto, Calif., USA). Although Ethos® is dedicated to oART only its non-adaptive mode was used in this study. Daily CBCT using the “pelvis fast” or “pelvis large fast” modes (acquisition time: 21.2 and 25 seconds, respectively) was acquired for image-guided RT. Two supplementary CBCTs were obtained during the first five RT sessions (short-course or long-course RT) and weekly thereafter (long-course RT) to evaluate the intrafraction motion of pelvic organs and target volumes.

### 3.1.2.3. Boost planning target volume definition

The  $PTV_{Boost}$  was defined using the van Herk's formalism [174]. Systematic and random geometrical treatment errors were integrated in a quadratic sum to derive the  $PTV_{Boost}$  margin:

$$PTV_{Boost} = \alpha\Sigma + \beta\sigma - \beta\sigma_p \text{ (Eq. 3.1.1)}$$

With  $\Sigma$  and  $\sigma$  as standard deviations of systemic and random errors, respectively,  $\sigma_p$  as the penumbra width, and  $\alpha = 2.5$ ,  $\beta = 1.64$  ensuring 90% of patients receive 95% of the dose [174]. For non-adaptive RT,  $PTV_{Boost}$  margin ( $PTV_{Non-ART}$ ) included the systematic errors related to tumour motion ( $\Sigma_{TM}$ ), setup ( $\Sigma_{SETUP}$ ), and delineation variability ( $\Sigma_D$ ); and random errors related to tumour motion ( $\sigma_{TM}$ ), setup ( $\sigma_{SETUP}$ ), and penumbra ( $\sigma_p$ ) [174, 295].

$$PTV_{Non-ART} = \alpha\sqrt{\Sigma_{TM}^2 + \Sigma_{SETUP}^2 + \Sigma_D^2} + \beta\sqrt{\sigma_{TM}^2 + \sigma_{SETUP}^2 + \sigma_p^2} - \beta\sigma_p \text{ (Eq. 3.1.2)}$$

While this formula applied to spherical, rigid tumour with tumour motion described by translation only, this was not applicable for the rectum [174, 295]. In this study, the  $CTV_{boost}$  is rectal segment, whose motion could be divided into translational ( $TMT$ ) and deformation ( $TMD$ ) components. Both  $TMT$  and  $TMD$  values approached a Gaussian distribution, for which a systematic and random error can be computed and added:

$$PTV_{Non-ART} = \alpha\sqrt{\Sigma_{TMT}^2 + \Sigma_{TMD}^2 + \Sigma_{SETUP}^2 + \Sigma_D^2} + \beta\sqrt{\sigma_{TMT}^2 + \sigma_{TMD}^2 + \sigma_{SETUP}^2 + \sigma_p^2} - \beta\sigma_p \text{ (Eq. 3.1.3)}$$

For oART  $PTV_{Boost}$  margin ( $PTV_{ART}$ ) computation, the daily delineation of organ-at-risks and target volumes, along with the computation of a new plan

for each session, eliminated patient setup variations ( $\Sigma_{SETUP}$  and  $\sigma_{SETUP}$ ). However, as a result of the daily CTV<sub>boost</sub> adjustments, we should incorporate a random component of the delineation variability error ( $\sigma_D$ ). Finally, since interfraction motion is compensated by oART, this interfraction motion (*ITM*) should be integrated in the non-adaptive margin, while when using daily oART, only the intrafraction motion (*iTM*) should be considered, which is minimal compared to interfraction motion [282]. Therefore, it has been assumed that integrating interfraction motion uncertainties in the PTV would also compensate for intrafraction motion in the non-adaptive strategy. By integrating all these errors, the formula for the non-adaptive PTV margin becomes:

$$PTV_{Non-ART} = \alpha \sqrt{\Sigma_{ITMT}^2 + \Sigma_{ITMD}^2 + \Sigma_{SETUP}^2 + \Sigma_D^2} + \beta \sqrt{\sigma_{ITMT}^2 + \sigma_{ITMD}^2 + \sigma_{SETUP}^2 + \sigma_p^2} - \beta \sigma_p \quad (Eq. 3.1.4)$$

And for the oART PTV margin:

$$PTV_{ART} = \alpha \sqrt{\Sigma_{iTM}^2 + \Sigma_{iTMD}^2 + \Sigma_D^2} + \beta \sqrt{\sigma_{iTM}^2 + \sigma_{iTMD}^2 + \sigma_D^2 + \sigma_p^2} - \beta \sigma_p \quad (Eq. 3.1.5)$$

All errors and margins were computed in the three dimensions, reflecting different rectal motion patterns along these axis [294, 295, 297-299] (See Appendix 6 for computation of these errors).

#### 3.1.2.4. Plan evaluation

Three different plans were generated and virtually delivered using an emulator of the Ethos' adaptive treatment planning system: (1) a Non-

adaptive plan using  $PTV_{Non-ART}$  with a boost dose of 2.16 Gy per fraction (Non-ART<sub>54Gy</sub>), (2) an adaptive plan using  $PTV_{ART}$  with a boost dose of 2.16 Gy per fraction (oART<sub>54Gy</sub>), and (3) an adaptive plan using  $PTV_{ART}$  with a boost dose of 2.4 Gy per fraction (oART<sub>60Gy</sub>). This emulator simulates the different adaptive radiotherapy steps: after the CBCT import from a database, the software performs an artificial intelligence-based delineation of some OARs called ‘influencers’ (in this study: the rectum, bladder, and bowel), which are reviewed and manually edited if inaccurate by a radiation oncologist. Then, target volumes are automatically propagated using a structure-guided deformable registration between planning CT and daily CBCT, which is driven by the influencer volumes. These target volumes are also evaluated and adjusted if needed, using the planning CT and MRI displayed on another screen. Finally, an adaptive plan is computed and compared with the non-adaptive initial plan, with the best option selected by a radiation oncologist. Aside from replacing the imported CBCT by real-time acquisition, all these steps are identical to clinical procedures. For Non-ART<sub>54Gy</sub>, the non-adaptive plan was always chosen; for oART<sub>54Gy</sub> and oART<sub>60Gy</sub>, the adaptive plan was systematically selected. For the purpose of this study, only the primary rectal tumour was boosted, without considering lymph nodes dose escalation. Patients with metastatic disease received radical radiotherapy for the pelvic tumour and lymph nodes, metastasis treatments were handled independently.

For all plans, 1.8 Gy per fraction was prescribed to the elective PTV ( $PTV_{Low}$ ), corresponding to a conventional dose of 45 Gy to pelvic lymph nodes based on international recommendations [161, 285]. Target volume goals and

organ-at-risk dose constraints were classified according to their priority (from 1 = most important to 3 = least important, Table 3.1.1).

| Dose/volume objectives for the whole treatment | Dose/volume objectives per session                     | Priority |
|------------------------------------------------|--------------------------------------------------------|----------|
| PTV Boost V95% > 98%                           | PTV Boost V <sub>95%</sub> > 98%                       | 1        |
| PTV Boost V105% < 2%                           | PTV Boost V <sub>105%</sub> < 2%                       |          |
| PTV Low V95% > 98%                             | PTV Low V <sub>95%</sub> > 98%                         |          |
| Small Bowel D0.1 cm <sup>3</sup> < 50 Gy       | Small Bowel D <sub>0.1 cm<sup>3</sup></sub> < 2 Gy     |          |
| Small Bowel V15 Gy < 200 cc                    | Small Bowel V <sub>0.6 Gy</sub> < 200 cc               | 2        |
| Small Bowel V45 Gy < 65 cc                     | Small Bowel V <sub>1.8 Gy</sub> < 65 cc                |          |
| Colon&Sigmoid D0.1 cm <sup>3</sup> < 54 Gy     | Colon&Sigmoid D <sub>0.1cm<sup>3</sup></sub> < 2.16 Gy |          |
| Bladder D35% < 40 Gy                           | Bladder D <sub>35%</sub> < 1.6 Gy                      |          |
| Bladder D5% < 50 Gy                            | Bladder D <sub>5%</sub> < 2 Gy                         |          |
| Bladder D50% < 35 Gy                           | Bladder D <sub>50%</sub> < 1.4 Gy                      |          |
| Femoral joint L/R D35% < 40 Gy                 | Femoral joint L/R D <sub>35%</sub> < 1.6 Gy            | 3        |
| Femoral joint L/R D5% < 50 Gy                  | Femoral joint L/R D <sub>5%</sub> < 2 Gy               |          |
| Femoral joint L/R D50% < 30 Gy                 | Femoral joint L/R D <sub>50%</sub> < 1.2 Gy            |          |
| Genitalia D35% < 30 Gy                         | Genitalia D <sub>35%</sub> < 1.2 Gy                    |          |
| Genitalia D5% < 40 Gy                          | Genitalia D <sub>5%</sub> < 1.6 Gy                     |          |
| Genitalia D50% < 20 Gy                         | Genitalia D <sub>50%</sub> < 0.8 Gy                    |          |

*Table 3.1.1: Dose/volume objectives classed according to the priority for plan generation. The small bowel is defined as bowel loops, genitalia encompass external genital organs (vulva in women; penis and testicles in men). L: Left, R: Right.*

Non-ART<sub>54Gy</sub>, oART<sub>54Gy</sub>, and oART<sub>60Gy</sub> plans were obtained for 33 virtual RT sessions (3 per patient) and compared according to their compliance with dose/volume objectives.

### 3.1.2.5. Tumour regression probability evaluation

The tumour regression probability (TRP) was compared between Non-ART<sub>54Gy</sub>, oART<sub>54Gy</sub>, and oART<sub>60Gy</sub> plans. The per-voxel CTV<sub>boost</sub> dose (*Dvoxel*) was introduced in the model developed by Applet et al., 2013 [162]. Briefly, this model integrated the *Dvoxel*, the tumour volume (*Tvol*, in cm<sup>3</sup>) and the *N-stage* to calculate TRP:

$$TRP_{voxel} = \frac{e^{-2.634 + 0.0349Dvoxel - 0.0058Tvol - 0.6252Nstage}}{1 + e^{-2.634 + 0.0349Dvoxel - 0.0058Tvol - 0.6252Nstage}} \quad (Eq. 3.1.6)$$

Coefficients were fixed to evaluate a TRP for a Tumour regression grade 1, corresponding to complete response without any residual tumour cell, for an actual delivered dose [162, 300]. N-stage and tumour volume were reported for each patient. For each individual fraction, tumour regression probability was calculated as the average of all TRP<sub>voxel</sub> values within the CTV<sub>boost</sub>.

### 3.1.2.6. Data analysis

Median values with interquartile range were reported and compared using the Wilcoxon signed-rank test. Statistical comparisons between plans were performed using a linear mixed model, with the patient as a random factor due to multiple observations per patient. Post-hoc pairwise comparisons between plans were performed and adjusted for multiple comparisons using the Benjamini-Hochberg method. A p-value < 0.05 was considered significant. Image analyses for error quantifications and tumour regression

probability calculation were performed using in-house developed Python (version 3.10) scripts on Visual Studio Code (version 1.18.1). All statistical analyses were performed on RStudio (R version 4.2.1) using the “tidyverse” package.

### **3.1.3. Results**

Demographic characteristics of the 11 included patients are reported in Table 3.1.2.



| Rectum RT patients                                    |               |
|-------------------------------------------------------|---------------|
| <b>N</b>                                              | 11            |
| <b>Gender</b>                                         |               |
| Male                                                  | 10            |
| Female                                                | 1             |
| <b>Age (mean +/- SD)</b>                              | 57.6 +/- 15.0 |
| <b>Tumour location</b>                                |               |
| Low rectum                                            | 3             |
| Middle rectum                                         | 5             |
| High rectum                                           | 3             |
| <b>T stage</b>                                        |               |
| T3                                                    | 8             |
| T4                                                    | 3             |
| <b>N stage</b>                                        |               |
| N0                                                    | 1             |
| N1                                                    | 3             |
| N2                                                    | 7             |
| <b>M stage</b>                                        |               |
| M0                                                    | 8             |
| M1                                                    | 3             |
| <b>Number CBCTs for interfraction motion analysis</b> | 95            |
| <b>Number CBCTs for intrafraction motion analysis</b> | 285           |

Table 3.1.2: Characteristics of the patients. CBCT: Cone-beam computed tomography, SD: Standard deviation.

Setup systematic and random error values were set to 0.5 and 1 mm, respectively, in all directions according to Nijkamp et al., 2009 [295]. The

penumbra was fixed at 2.9 mm [301]. Tumour motion errors, delineation errors, and final PTV margins for non-adaptive and oART strategies are reported in Table 3.1.3.

|                                               | Antero-posterior | Left-right | Supero-inferior |
|-----------------------------------------------|------------------|------------|-----------------|
| <b>Tumour motion translation (<i>TMT</i>)</b> |                  |            |                 |
| Interfraction ( <i>ITMT</i> )                 |                  |            |                 |
| $\Sigma$                                      | 4.2              | 1.1        | 2.5             |
| $\sigma$                                      | 3.6              | 1.4        | 3.4             |
| Intrafraction ( <i>iTMT</i> )                 |                  |            |                 |
| $\Sigma$                                      | 0.6              | 0.3        | 0.7             |
| $\sigma$                                      | 1.5              | 0.8        | 1.6             |
| <b>Tumour motion deformation (<i>TMD</i>)</b> |                  |            |                 |
| Interfraction ( <i>ITMD</i> )                 |                  |            |                 |
| $\Sigma$                                      | 1.1              | 0.5        | 1.0             |
| $\sigma$                                      | 0.6              | 0.4        | 1.4             |
| Intrafraction ( <i>iTMD</i> )                 |                  |            |                 |
| $\Sigma$                                      | 0.6              | 0.3        | 0.6             |
| $\sigma$                                      | 0.4              | 0.2        | 1.7             |
| <b>Delineation (<i>D</i>)</b>                 |                  |            |                 |
| $\Sigma$                                      | 0.6              | 1.1        | 2.5             |
| $\sigma$                                      | 0.4              | 0.4        | 1.9             |
| <b>Setup</b>                                  |                  |            |                 |
| $\Sigma$                                      | 0.5              | 0.5        | 0.5             |
| $\sigma$                                      | 1.0              | 1.0        | 1.0             |
| <b>Penumbra (<i>p</i>)</b>                    |                  |            |                 |
|                                               | 2.9              | 2.9        | 2.9             |
| <b>PTV margins</b>                            |                  |            |                 |
| <b>PTV<sub>Non-ART</sub></b>                  | 14.2             | 5.0        | 12.3            |
| <b>PTV<sub>ART</sub></b>                      | 3.3              | 3.2        | 8.7             |

Table 3.1.3: Systematic ( $\Sigma$ ) and random ( $\sigma$ ) error values in mm for the tumour motion, delineation variability, and setup that are integrated in the total PTV margin. ART: Adaptive radiotherapy, PTV: Planning target volume

The median  $PTV_{Non-ART}$  volume was  $214.3 \text{ cm}^3$  ( $188.1 \text{ cm}^3 - 341.8 \text{ cm}^3$ ), significantly larger than the median  $PTV_{ART}$  volume of  $122.5 \text{ cm}^3$  ( $108.3 \text{ cm}^3 - 209.8 \text{ cm}^3$ ;  $p < 0.001$ , Wilcoxon paired; Figure 3.1.1).

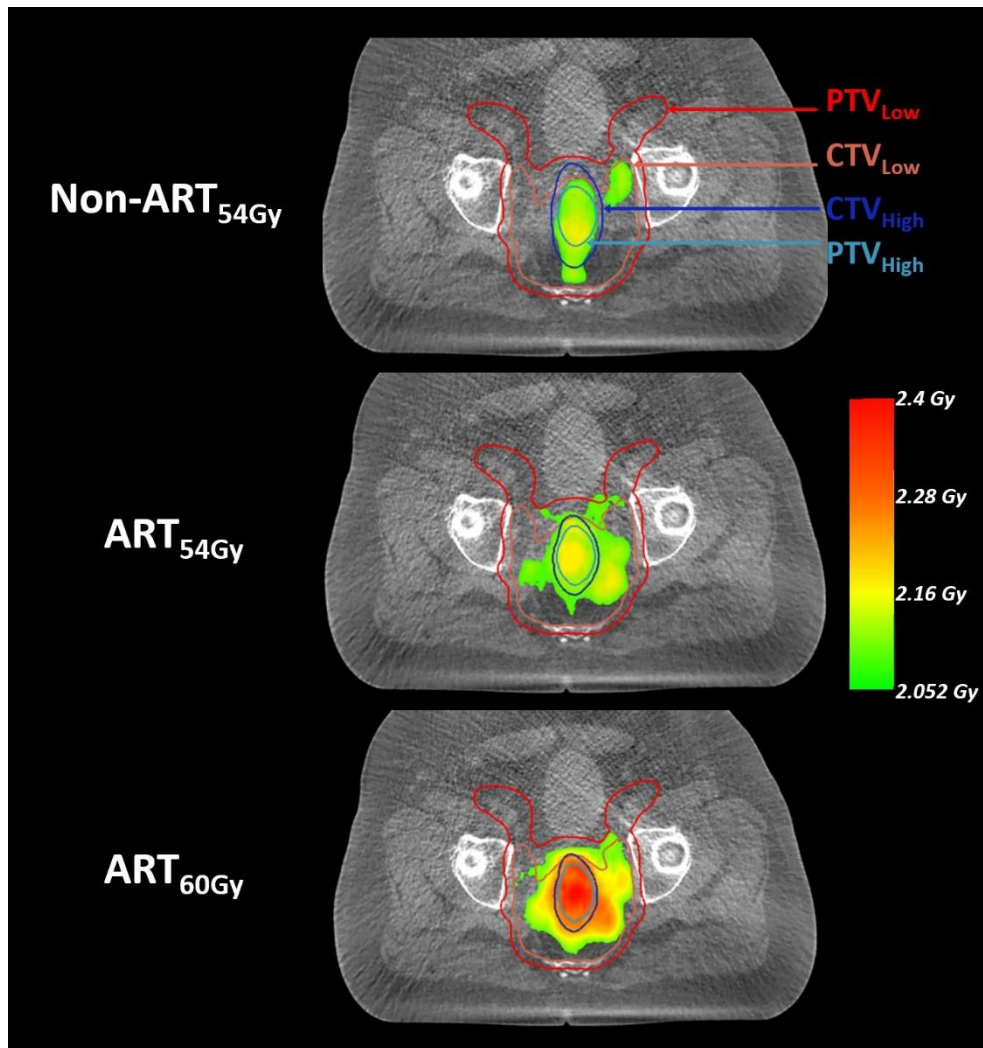


Figure 3.1.1: Comparison between  $PTV_{Non-ART}$  and  $PTV_{ART}$  volume and dose distribution of Non-ART<sub>54Gy</sub>, ART<sub>54Gy</sub>, and ART<sub>60Gy</sub>. The dark blue and dark red lines are the  $PTV_{Boost}$  and the  $PTV_{Low}$ , respectively. The light blue and light red lines are the  $CTV_{Boost}$  and the  $CTV_{Low}$ , respectively.

*PTVNon-ART was used only for Non-ART54Gy plans and is significantly bigger ( $p < 0.0001$ ) than the PTVART used for ART54Gy and ART60Gy plans. The isodose levels 2.052 Gy and 2.16 Gy correspond to 95% and 100% respectively of the prescribed dose to the primary tumour in plans Non-ART54Gy, and ART54Gy. The isodose levels 2.28 Gy and 2.4 Gy correspond to 95% and 100% respectively of the prescribed dose to the primary tumour in plan ART60Gy. Coverage of the PTVBoost by the 95% isodose is achieved with ART54Gy, or ART60Gy, but not with Non-ART54Gy. ART: Adaptive radiotherapy, CTV: Clinical target volume, PTV: Planning target volume.*

Among the 33 fractions delivered for each plan, the most frequently violated dose/volume goal was the small bowel volume receiving over 15 Gy (priority 2), for the ART<sub>54Gy</sub> and the ART<sub>60Gy</sub> plans ( $n = 20/33$  and  $19/33$ , respectively). For the Non-ART<sub>54Gy</sub>, the most frequent violation was the PTV<sub>Boost</sub> volume receiving more than 95% of the prescribed dose (priority 1). Median PTV<sub>Boost</sub> and PTV<sub>Low</sub> volumes covered by at least 95% of the prescribed dose were significantly lower with Non-ART<sub>54Gy</sub> plan compared to ART<sub>54Gy</sub> and ART<sub>60Gy</sub> ( $p < 0.001$  in each case). There were 70/132, 20/132, and 18/132 priority 1 dose/volume objective violations for Non-ART<sub>54Gy</sub>, ART<sub>54Gy</sub>, and ART<sub>60Gy</sub> plans, respectively. Violations occurred for 45/198 priority 2 dose/volume objectives in Non-ART<sub>54Gy</sub> plans, 33/198 in ART<sub>54Gy</sub> plans, and 34/198 in ART<sub>60Gy</sub> plans (Table 3.1.4).

| Dose/volume objectives<br>per RT session      | Priority | Non-ART <sub>54Gy</sub> |                                       | oART <sub>54Gy</sub> |                                       |                                            | oART <sub>60Gy</sub> |                                       |                                            |                                         |
|-----------------------------------------------|----------|-------------------------|---------------------------------------|----------------------|---------------------------------------|--------------------------------------------|----------------------|---------------------------------------|--------------------------------------------|-----------------------------------------|
|                                               |          | N<br>violations         | Median value<br>[interquartile range] | N<br>violations      | Median value<br>[interquartile range] | Comparison with<br>Non-ART <sub>54Gy</sub> | N<br>violations      | Median value<br>[interquartile range] | Comparison with<br>Non-ART <sub>54Gy</sub> | Comparison<br>with oART <sub>54Gy</sub> |
| PTV Boost V <sub>95%</sub> > 98%              | 1        | 29                      | 93.3% [81.4% - 97.3%]                 | 4                    | 98.9% [98.6% - 99.4%]                 | ****                                       | 4                    | 98.9% [98.6% - 99.0%]                 | ****                                       | ns                                      |
| PTV Boost V <sub>105%</sub> < 2%              |          | 7                       | 0.0% [0.0% - 1.1%]                    | 0                    | 0.0% [0.0% - 0.0%]                    | ns                                         | 0                    | 0.0% [0.0% - 0.0%]                    | ns                                         | ns                                      |
| PTV Low V <sub>95%</sub> > 98%                |          | 17                      | 98.0% [96.2% - 98.9%]                 | 0                    | 99.8% [99.8% - 99.9%]                 | ****                                       | 0                    | 99.9% [99.8% - 99.9%]                 | ****                                       | ns                                      |
| Small Bowel D <sub>0.1 cm³</sub> < 2 Gy       |          | 17                      | 2.01 Gy [1.95 Gy - 2.21 Gy]           | 16                   | 1.99 Gy [1.90 Gy - 2.17 Gy]           | ns                                         | 14                   | 1.97 Gy [1.92 Gy - 2.21 Gy]           | ns                                         | ns                                      |
| Small_Bowel V <sub>0.6 Gy</sub> < 200 cc      | 2        | 20                      | 240 cm³ [141 cm³ - 459 cm³]           | 20                   | 222 cm³ [169 cm³ - 347 cm³]           | ns                                         | 19                   | 222 cm³ [173 cm³ - 360 cm³]           | ns                                         | ns                                      |
| Small_Bowel V <sub>1.8 Gy</sub> < 65 cc       |          | 7                       | 26.3 cm³ [6.2 cm³ - 45.2 cm³]         | 6                    | 14.0 cm³ [4.2 cm³ - 43.3 cm³]         | ns                                         | 6                    | 15.4 cm³ [6.6 cm³ - 41.8 cm³]         | ns                                         | ns                                      |
| Colon&Sigmoid D <sub>0.1 cm³</sub> < 2.16 Gy  |          | 5                       | 1.78 Gy [0.70 Gy - 2.10 Gy]           | 6                    | 1.85 Gy [82 Gy - 2.14 Gy]             | ns                                         | 6                    | 1.83 Gy [0.80 Gy - 2.05 Gy]           | *                                          | ns                                      |
| Bladder D <sub>35%</sub> < 1.6 Gy             |          | 3                       | 1.25 Gy [1.10 Gy - 1.36 Gy]           | 0                    | 1.09 Gy [0.85 Gy - 1.29 Gy]           | ns                                         | 1                    | 1.24 Gy [0.94 Gy - 1.42 Gy]           | ns                                         | ns                                      |
| Bladder D <sub>5%</sub> < 2 Gy                |          | 8                       | 1.81 Gy [1.57 Gy - 1.98 Gy]           | 1                    | 1.78 Gy [1.70 Gy - 1.89 Gy]           | ns                                         | 2                    | 1.81 Gy [1.75 Gy - 1.86 Gy]           | ns                                         | ns                                      |
| Bladder D <sub>50%</sub> < 1.4 Gy             |          | 2                       | 1.07 Gy [0.96 Gy - 1.21 Gy]           | 0                    | 0.80 Gy [0.67 Gy - 1.13 Gy]           | **                                         | 0                    | 1.09 Gy [0.80 Gy - 1.25 Gy]           | ns                                         | **                                      |
| Femoral joint Left D <sub>35%</sub> < 1.6 Gy  | 3        | 0                       | 0.61 Gy [0.55 Gy - 0.71 Gy]           | 0                    | 0.66 Gy [0.54 Gy - 0.69 Gy]           | ns                                         | 0                    | 0.67 Gy [0.62 Gy - 0.74 Gy]           | *                                          | *                                       |
| Femoral joint Right D <sub>35%</sub> < 1.6 Gy |          | 0                       | 0.63 Gy [0.55 Gy - 0.67 Gy]           | 0                    | 0.59 Gy [0.49 Gy - 0.67 Gy]           | ns                                         | 0                    | 0.65 Gy [0.61 Gy - 0.70 Gy]           | ns                                         | *                                       |
| Femoral joint Left D <sub>5%</sub> < 2 Gy     |          | 0                       | 1.06 Gy [0.95 Gy - 1.11 Gy]           | 0                    | 1.01 Gy [0.92 Gy - 1.08 Gy]           | ns                                         | 0                    | 1.06 Gy [0.95 Gy - 1.12 Gy]           | ns                                         | ns                                      |
| Femoral joint Right D <sub>5%</sub> < 2 Gy    |          | 0                       | 1.07 Gy [0.88 Gy - 1.11 Gy]           | 0                    | 0.96 Gy [0.90 Gy - 1.06 Gy]           | ns                                         | 0                    | 1.03 Gy [0.94 Gy - 1.08 Gy]           | ns                                         | ns                                      |
| Femoral joint Left D <sub>50%</sub> < 1.2 Gy  |          | 0                       | 0.52 Gy [0.31 Gy - 0.57 Gy]           | 0                    | 0.49 Gy [0.43 Gy - 0.55 Gy]           | ns                                         | 0                    | 0.52 Gy [0.42 Gy - 0.56 Gy]           | ns                                         | ns                                      |
| Femoral joint Right D <sub>50%</sub> < 1.2 Gy |          | 0                       | 0.47 Gy [0.27 Gy - 0.57cGy]           | 0                    | 0.44 Gy [0.33 Gy - 0.50 Gy]           | ns                                         | 0                    | 0.43 Gy [0.34 Gy - 0.50 Gy]           | ns                                         | ns                                      |
| Genitalia D <sub>35%</sub> < 1.2 Gy           |          | 0                       | 0.14 Gy [0.11 Gy - 0.21 Gy]           | 0                    | 0.11 Gy [0.9 Gy - 0.19 Gy]            | ns                                         | 0                    | 0.13 Gy [0.09 Gy - 0.20 Gy]           | ns                                         | ns                                      |
| Genitalia D <sub>5%</sub> < 1.6 Gy            |          | 1                       | 0.49 Gy [0.30 Gy - 0.56 Gy]           | 0                    | 0.48 Gy [0.25 Gy - 0.53 Gy]           | ns                                         | 0                    | 0.39 Gy [0.30 Gy - 0.64 Gy]           | ns                                         | ns                                      |
| Genitalia D <sub>50%</sub> < 0.8 Gy           |          | 0                       | 0.08 Gy [0.08 Gy - 0.16 Gy]           | 0                    | 0.08 Gy [0.07 Gy - 0.11 Gy]           | ns                                         | 0                    | 0.08 Gy [0.06 Gy - 0.15 Gy]           | ns                                         | ns                                      |

*Table 3.1.4: Dose/volume results of the 33 RT sessions delivered. Comparisons between values of each plan were performed using a linear mixed effect model with post-hoc analysis and correction for multiple comparisons using the Benjamini-Hochberg method. ns: Not significant, \* p-value < 0.05, \*\* p-value < 0.01, \*\*\*\* p-value < 0.001. (oA)RT: (Online-adaptive) radiotherapy.*

The median tumour regression probability value was 16.9% [12.6% - 19.0%] for Non-ART<sub>54Gy</sub>, 17.0% [12.7% – 19.1%] for ART<sub>54Gy</sub>, and 20.8% [15.7% - 23.3%] for oART<sub>60Gy</sub>. These values were significantly higher for oART<sub>60Gy</sub> compared to the two other plans (linear mixed effect model,  $p < 0.001$ ), but not significantly different between Non-ART<sub>54Gy</sub> and oART<sub>54Gy</sub> ( $p = 0.16$ ; Figure 3.1.2).

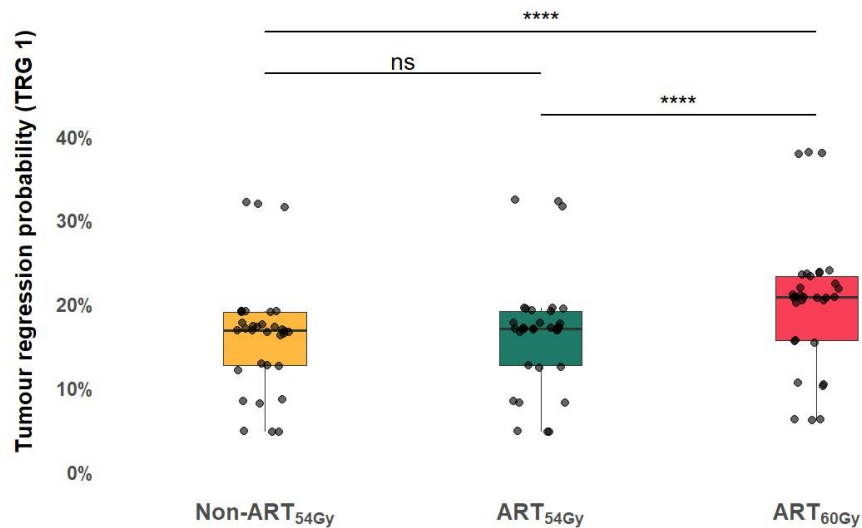


Figure 3.1.2: Tumour regression probability comparison between Non-ART<sub>54Gy</sub>, ART<sub>54Gy</sub>, and ART<sub>60Gy</sub> based on the dose-response model developed by Appelt et al. 2013 [162]. \*\*\*\*  $p < 0.0001$ . ART: Adaptive radiotherapy, TRG: Tumour regression grade.

### 3.1.4. Discussion

In this study, we proposed a population-based PTV definition for both non-adaptive and CBCT-guided online-adaptive RT (oART) strategies for rectal boost. Unlike non-adaptive strategies, a random delineation error was



added to the systematic delineation error since the CTV<sub>Boost</sub> is delineated daily, potentially by different operators [283]. Online adaptation allowed a PTV margin reduction of 10.9, 1.8, and 3.6 mm in the antero-posterior, left-right, and supero-inferior directions, respectively. Margins were larger in the antero-posterior and supero-inferior axes, consistent with other population-based PTV margin for rectal tumour [296, 302, 303]. Tumour motion (translation and deformation) accounted for most of the PTV margin. In the anterior-posterior axis, interfraction translation is much higher than the intrafraction translation motion, leading to an important difference between the non-adaptive and oART antero-posterior PTV margin. In addition to PTV reduction, oART (oART<sub>54Gy</sub> and oART<sub>60Gy</sub>) achieved better target volume coverage than non-adaptive RT (Non-ART<sub>54Gy</sub>) with a similar risk of organ-at-risk expected toxicities. With non-adaptive strategy, the PTV<sub>Boost</sub> coverage objective was not met in 87.9% of sessions, compared to only 12.1% with oART. The PTV<sub>Low</sub> coverage was always achieved in oART sessions but only in 48.5% of non-adaptive sessions. Regarding priority 1&2 OAR objectives, more violations occurred (even not significant,  $p = 0.32$ ) with Non-ART<sub>54Gy</sub> (62/231) compared to oART<sub>54Gy</sub> (49/231), and oART<sub>60Gy</sub> (48/231). Furthermore, oART enabled dose-escalation, significantly increasing the tumour regression probability without increasing toxicity risk. These results suggest that oART could increase the proportion of patients eligible for organ preservation strategy and, therefore, improve quality of life.

Additional strategies may further decrease PTV margins. For example, multi-modality imaging such as T2- or diffusion-weighted MRI and F-18 fluorodeoxyglucose positron emission tomography improves interoperator

agreement in delineation [304-306]. Also, MRI-guided RT allows direct visualisation of the tumour and real-time ART. By tracking the tumour or repeating multiple non-irradiating images with the MRI system associated with a linear accelerator, the beam could be stopped when the tumour extends spatial tolerance limits to enable plan adaptation [166, 302, 307-309]. Kensen et al., 2023 reported PTV margins for MRI-guided boost of the primary tumour of 3.2, 5.6, 1.7, and 4.7 mm in the anterior, posterior, left-right, and supero-inferior directions, respectively [302]. While these values are even lower than ours, their boost volume included only the GTV, for which the tumour motion can be described only by the translation of the centre of mass of the tumour without considering the deformation component, and delineation errors [302]. Moreover, MRI-guided RT systems availability is limited, similarly to machines dedicated to CBCT-guided oART, due important financial, time and human resources required [187].

The PTV reduction with CBCT-guided oART opens the door for more preservative treatments considering the dose-response relationship demonstrated in locally advanced colorectal cancer [162]. Also, high pathological complete response rates with acceptable toxicities have been highlighted when increasing the dose to the primary rectal tumour above 60 Gy [163]. Therefore, delivering a higher dose to the primary tumour should be investigated in addition to the total neoadjuvant therapy, which already allows rectal preservation in up to 50% of the patients [103, 162]. One of the major limitations for dose escalation remains the dose delivered to the pelvic OARs. This could lead to an excess of RT-induced adverse events and a reduction of the quality of life compared with standard treatment [165].

By reducing the PTV margin, CBCT-guided oART could reduce the dose received by OARs and, therefore, possibly reduce the adverse events.

This study has limitations. First, the small number of patients limits the inclusion of other important parameters into our PTV margins. The patient gender, the treatment position, the treatment progress over time, the tumour location in the rectum, or the patient preparation can influence the rectal motion and, thus, the PTV margin values [292-296, 310-312]. Particularly, there is an imbalance between male ( $n = 10$ ) and female ( $n = 1$ ), potentially underestimating PTV margin size in women, who present a higher systematic variation in the mesorectum shape [311]. Also, rectal motion is greatly impacted by session duration [166]. Kleijnen et al., 2016 used a cine-MRI in 16 patients for rectal tumour motion quantification. They reported that the intrafraction motion amplitude is similar interfraction motion amplitude after only 18 minutes [166]. While session duration is critical for oART, the workflow presented here should be adapted to reduce this duration as much as possible and limit the intrafraction motion impact [194, 313, 314]. Second, we used a rectal boost volume including the circumferential rectal wall, following major rectum RT trials [103, 288]. In the absence of any consensus regarding boost delineation, PTV<sub>boost</sub> margins presented here could not be applicable for other teams, using different CTV<sub>boost</sub> definitions. Third, to homogenise the cohort, no RT boost was delivered to lymph node. While nodal boosts are common in practice, their PTV margin should be computed individually, as uncertainties may differ from those of the primary tumour. Fourth, the tumour regression probability model used, published in 2013, does not account for recent developments

in LARC management, such as induction/consolidation chemotherapy or new molecular classifications predictive of treatment response [102, 103, 115, 315]. Also, contemporary tumour regression probability results obtained after total neoadjuvant therapy are higher than those expected here [102, 103, 115]. Thus, tumour regression probability findings should be interpreted cautiously, focusing on relative improvements from dose escalation rather than absolute values.

External-beam RT for rectal boost lacks clear consensus regarding the definition of the volumes to be irradiated. The GTV<sub>boost</sub> alone or added with a numeric or anatomical margin (such as the rectum or the mesorectum) could be considered an acceptable CTV<sub>boost</sub> [103, 288-290, 316]. Definitions of the PTV are even more variable among different studies [103, 289, 290, 292, 316]. Using an empirical isotropic margin is widespread, often in the absence of an experimental or clinical rationale [103, 289, 290]. More comprehensive PTV definitions that compensate for interfraction rectal motion have also been described [292-294]. Other teams also proposed a PTV definition that integrates set-up and/or delineation errors in addition to the rectal motion [295, 296]. However, these suggested margins remain considerable and their use results in a large total irradiated volume, which could lead to potential RT-induced toxicities for pelvic OARs.

In conclusion, we present a population-based PTV margin quantification for rectal boost RT. Compared to non-adaptive strategies, CBCT-guided online-adaptive RT allowed a reduction of this margin eliminating interfraction motion errors. Due to larger tumour motion, PTV margins were greater in

the antero-posterior and supero-inferior axes, respectively, compared to the left right axis. This PTV reduction combined with daily adaptation allows a better target volume coverage with similar dose to OARs compared to non-adaptive strategies. Furthermore, when escalating the primary tumour dose, a better tumour control probability is expected with, again, a similar toxicity risk compared to non-adaptive strategy. Online-adaptive RT opens the door for a safe dose-escalation to the primary rectal tumour and, therefore, offering potentially more organ preservation in an increasing proportion of locally advanced rectal cancer patients.

### **3.2. Radiotherapy boost to the primary tumour in locally advanced rectal cancer: Systematic review of practices and Meta-analysis**

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## Highlights

- Primary tumour RT boost is common practice in locally advanced rectal cancer
- High variability exists in techniques used for external radiotherapy rectal boost
- IMRT/VMAT, simultaneous boost, and dose escalation improve pathological CR
- No RT parameters are associated with clinical complete response and local recurrence rate, while data are limited
- Rectal boost radiotherapy guidelines are urgently required to standardise practice

## **Abstract**

### *Introduction*

In locally advanced rectal cancer (LARC), increasing the complete response rate after total neo-adjuvant treatment may increase the patient eligibility for non-operative management («Watch and Wait»). Although a radiotherapy (RT) boost to the primary tumour may enhance CR rates, clear guidelines are currently lacking. This systematic review and meta-analysis investigate the technical parameters used for rectal boost RT and assess their impact on oncological outcomes.

### *Methods*

Following PRISMA guidelines, the terms “rectum,” “radiotherapy,” and “boost” were searched in PubMed and EMBASE (PROSPERO: CRD42023444685). Studies reporting on external beam RT boost to the primary tumour in LARC and meeting quality criteria were included. Descriptive analyses extracted data on RT technique, preparation, boost delineation, dose, chemotherapy, and follow-up. A mixed-effects meta-analysis model evaluated the impact of selected parameters on complete response and local recurrence rate. Studies were analysed separately based on treatment intent: planned surgery or «Watch and Wait».

### *Results*

Out of 3904 references, 83 were included in the descriptive analysis and 78 in the meta-analysis. Substantial variability in RT parameters was observed across studies. Pathologic complete response rates were significantly higher with intensity-modulated/volumetric-modulated arc RT (IMRT/VMAT,



p=0.007), simultaneous boost (p=0.020), dose escalation (Biological equivalent dose > 74Gy, equivalent to >53 Gy in 2 Gy fractions, p=0.035), and the combination of induction and consolidation chemotherapy (p=0.023). No significant associations were found for clinical complete response or local recurrence rate.

### *Conclusion*

While rectal RT boost is already part of real-world practices, the wide heterogeneity in techniques highlights the urgent need for standardisation. Our meta-analysis suggests that IMRT/VMAT, simultaneous boost, and dose escalation are associated with higher pathological complete response rates and should be considered in future rectal boost guidelines. These findings, however, warrant careful interpretation due to the absence of adjustments for clinical, tumoral, or patient-related parameters that may also influence response rate and oncological outcomes.

### **3.2.1. Introduction**

Rectal cancer is the 8<sup>th</sup> most common malignancy worldwide and accounts for approximatively 344 000 deaths annually [317]. In locally advanced rectal cancer (LARC, defined as cT3-cT4 or N+ M0 disease), Total Neo-adjuvant Treatment (TNT) has emerged as a treatment option for selected patients, before surgical resection [94]. TNT consists of a preoperative combination of induction or consolidation chemotherapy with radio(chemo)therapy [94, 102, 115]. In recent years, the «Watch and Wait» strategy after TNT has also emerged as an interesting approach. This «Watch and Wait» strategy offers a non-operative management with active surveillance for patients achieving a clinical complete response after TNT, with the aim of preserving the rectum and improve quality of life [103, 121, 287]. During radiotherapy (RT), dose escalation to the primary tumour, or “boost”, can be delivered via external-beam RT or contact x-ray brachytherapy [94, 103, 121, 164]. This boost has shown the potential to improve complete response (CR) rates [162, 163]. However, its benefit, particularly for external-beam RT boost, has not been confirmed in prospective trials and may be ironically associated with a transient decline in quality of life [164, 165]. Further investigation is therefore warranted. While international recommendations exist for pelvic lymph node RT in LARC, no consensus is currently available for the primary rectal boost [161]. Before such guidelines can be established, a comprehensive understanding of the different techniques used for this RT boost and their impact is essential.

The first objective of this systematic review is to provide a descriptive analysis of the technical approaches used for RT boost planning across institutions. The second objective is to evaluate, through meta-analysis, the impact of these technical parameters on CR rates and local recurrence rates. Overall, this work aims to provide the foundation for future consensus guidelines on rectal boost RT.

### **3.2.2. Methods**

This systematic review and meta-analysis were conducted in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [318]. The protocol was published in the PROSPERO database (CRD42023444685).

#### ***3.2.2.1. Search strategy***

MEDLINE and Embase were searched using different combinations of the terms “rectum”, “radiotherapy”, “radiation therapy”, “boost”, and “dose escalation”. The initial search covered studies published from January 2000 to May 2023. The search was relaunched to include publications until June 14, 2024. Duplicate records were removed.

#### 3.2.2.2. *Studies selection*

Titles and abstracts were screened independently by two operators (J.P. and G.V.O.) using the PICOS (population, intervention, control, outcome, study design) approach [319]. Studies were eligible for full-text screening if they involve (P) human patients with rectal adenocarcinoma treated with (IC) a neo-adjuvant RT rectal boost/dose escalation in the primary setting. Analysed outcomes (O) are described in the next section. Retrospective, prospective, *in silico* studies, and study protocols (S) were included. Exclusion criteria included non-human studies, other cancers, intraoperative or postoperative boosts, non-English/French language, and inaccessible full texts. Conflicts were resolved by consensus. Full-text screening was conducted by one operator (J.P.) using the same eligibility criteria. Additionally, studies using only brachytherapy or contact X-ray therapy for the boost, as well as conference abstracts, were excluded. Reference lists of review articles were screened to identify any relevant studies not captured by the initial search. Aggregate data were extracted by J.P., using only publicly available information; no attempt was made to contact authors for missing data.

#### 3.2.2.3. *Boost descriptive analysis*

The descriptive analysis collected data on publication details, RT technique, delivery modalities, patient preparation, boost delineation, dose, concomitant chemotherapy, neo-adjuvant chemotherapy (induction and/or consolidation), and treatment strategy. Due to anticipated interstudy

heterogeneity, data were categorised (Table A7.5). RT doses were converted into biologically effective dose using an alpha/beta ratio of 5.06 (Appendix 7.1) [320].

When multiple studies from the same institution were identified, only the most recent was retained to better reflect current clinical practices.

#### *3.2.2.4. Meta-analysis*

The primary endpoint was the CR rate across subgroups defined in the descriptive analysis, including publication year, RT technique, clinical target volume (CTV) definition, planning target volume (PTV) margin, boost sequence, boost biologically effective dose, concomitant chemotherapy, neo-adjuvant chemotherapy, and surgical delay. Studies with planned surgery reporting pathological complete response rate were analysed separately from «Watch and Wait» studies reporting clinical complete response. A tumour regression grade 1 was considered equivalent to a pathological complete response [321]. Given the expected limited number of eligible «Watch and Wait» cohorts, the corresponding analysis is presented separately (Appendix 7.2).

The secondary endpoint was the local recurrence rate, within the same subgroups as CR analysis and based on the time-to-event data available in each study. In planned surgery cohorts, local recurrence rate was defined as the number of pelvic recurrences among all included patients. In «Watch and Wait» studies, local recurrence rate included pelvic recurrences in both

patients undergoing immediate surgery for non-clinical complete response and those managed non-operatively after achieving an initial clinical complete response.

Only studies consistently reporting or enabling the direct calculation of intention-to-treat results were included. When a single study reported multiple independent cohorts, based on differences in analysed factors, each cohort was analysed separately, provided CR outcomes were reported individually.

#### *3.2.2.5. Quality assessment*

A modified Newcastle-Ottawa Scale adapted to this study purpose was used for quality evaluation [322]. Eight items were scored by one operator (J.P.) from “A” (high quality) to “C” (poor quality, Table A7.6). For the descriptive analysis, studies were excluded if one of the two first items (“Complete RT technique description” and “Correct terminology”) scored “C”. For the meta-analysis, exclusion applied if any item scored “C”.

#### *3.2.2.6. Statistical analysis*

Wilcoxon signed-rank tests were used for comparisons of ordered variables. For the meta-analysis, pooled estimates of CR and local recurrence rate were presented as forest plots with 95% confidence interval, using the Knapp-Hartung adjustment [323]. The between-study heterogeneity variance ( $\tau^2$ ) was evaluated using the maximum likelihood estimator [324, 325]. For

subgroup containing fewer than five studies, a pooled  $\tau^2$  was calculated [326]. Statistical heterogeneity was quantified using the  $I^2$  statistic [327]. Subgroup comparisons were performed using a mixed-effects model using the Q-test, without adjustment for confounding factors. Given the study objective of identifying parameters associated with CR and local recurrence rate, only subgroup meta-analyses were reported, and no overall pooled estimates were provided.

A meta-regression model was conducted to explore the relationship between the RT boost dose and pathological complete response rate (Appendix 7.3).

Publication bias was assessed by visual inspection of funnel plots and Peters' tests [328, 329].

For all statistical analyses, a p-value < 0.05 was considered significant. All analyses were performed in RStudio (R version 4.2.1.) using the "meta" (v7.0-0), "dmetar" (v0.1.0), and "nls2" (v0.3-4) packages [330].

### **3.2.3. Results**

#### **3.2.3.1. Descriptive analysis**

The final search (June 14, 2024) identified 3904 references. After duplicate removal, 3050 unique records remained. Of these, 2378 were excluded by both operators, with consensus achieved for 110 studies. A total of 593 references were selected for full-text assessment. After including references identified from reviews, 211 full papers were included in the systematic

review (Figure 3.2.1, Figure A7.11, and Figure A7.12). Four studies reported two different RT boost dose levels; only the highest was considered for analysis [331-334].

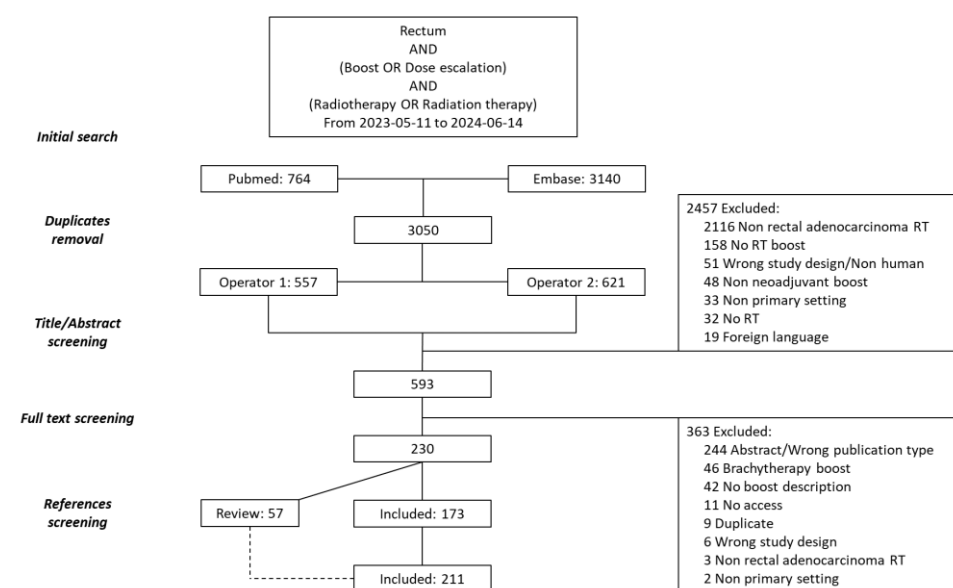


Figure 3.2.1: Flow chart of study selection. RT: Radiotherapy.

After institutional duplicate removal, 115 studies were included for quality assessment. Among these, 22 and 29 studies were rated “C” for the quality criteria “Complete RT description” and “Correct terminology”, respectively; 19 of them received a “C” for both criteria (Figure A7.13). Ultimately, 83 studies including 8060 patients were included in the descriptive analysis (Table A7.7 and Table A7.8) [103, 113, 121, 307, 331-408]. There were 39 prospective, 23 retrospective, 18 *in silico* studies, and 3 protocols included (Table A7.7). Detailed references are reported in Table A7.7 for clarity.



#### 3.2.3.1.1. RT technique: IMRT/VMAT more frequently used than 3D in recent studies.

Three-dimensional (3D) RT was used exclusively in 28.9% (24/83) of studies, while intensity-modulated RT (IMRT)/volumetric-modulated arc RT (VMAT) was used in 38.6% (32/83). The median publication year was significantly lower for studies using 3D compared to those using IMRT/VMAT (2012 vs. 2019,  $p < 0.001$ ). Magnetic resonance imaging (MRI)-guided RT was used by 4.8% (4/83) of teams. IMRT/VMAT and 3D were both used in 16.9% (14/83) of studies. Proton therapy was investigated in 2.4% (2/83) of studies. The RT technique was not reported in 9.6% (8/83) of studies.

#### 3.2.3.1.2. RT boost delivery modalities: sequential and simultaneous boosts are equally common; adaptive RT is emerging.

The boost was delivered sequentially in 42.2% (32/83) and simultaneously in another 42.2% (32/83) of publications, with no significant difference in the median publication year (2016 vs. 2019,  $p = 0.09$ ). Both approaches were used in 9.6% (8/83), and 3.6% (3/83) described mixed sequential/simultaneous boosts. The delivery modality was not specified in 2.4% (2/83) of studies. Four teams delivered the boost during a second daily session on selected days [363, 365, 394, 409]; one used bifractionated sequential boost [383], and another reported bifractionated RT for the entire treatment course [382]. Since 2018, adaptive RT for rectal boost

delivery has been reported by nine teams [307, 334, 347, 358, 364, 376, 377, 397, 404].

3.2.3.1.3. RT preparation and setup: bladder filling protocols and supine positioning are advised, but high variability remains.

Treatment positioning and bladder preparation were the most frequently reported parameters, mentioned in 67.5% (56/83) and 43.4% (36/83) of studies, respectively. Details on oral or rectal preparation and the use of intravenous contrast for planning CT were less commonly reported (Figure 3.2.2A).

Bladder protocols were multiple but the most frequent approaches involved a full bladder (18.1%, 16/83) or water intake (16.9%, 15/83, Figure 3.2.2B).

Rectal preparations, reported in 14.5% (12/83), also varied widely: rectal emptying [335, 355, 362], filling [372, 390], opacification [344, 393], gel use [347], or no specific preparation in 4 studies [338, 358, 404, 406] (Figure 3.2.2C).

Oral contrast administration for planning CT was mentioned in 13.3% (11/83) of studies, with one specifying water as the contrast agent [339]; three studies explicitly reported not using oral contrast (Figure 3.2.2D) [358, 403, 406].

Intravenous contrast for planning CT acquisition was mentioned in 10.8% (9/83) of studies; one explicitly stating its absence [332] (Figure 3.2.2E).

When studies reported the treatment position, 30.1% (25/83) used the prone position and 26.5% (22/83) used supine. Supine positioning was more common in recent publications (median publication year 2020 vs. 2016 for prone,  $p < 0.001$ , Figure 3.2.2F).

Other rare preparation techniques included use anal radiopaque marker during planning CT acquisition [113, 385, 388], pre-rectal hydrogel injection [335], and fasting [401].

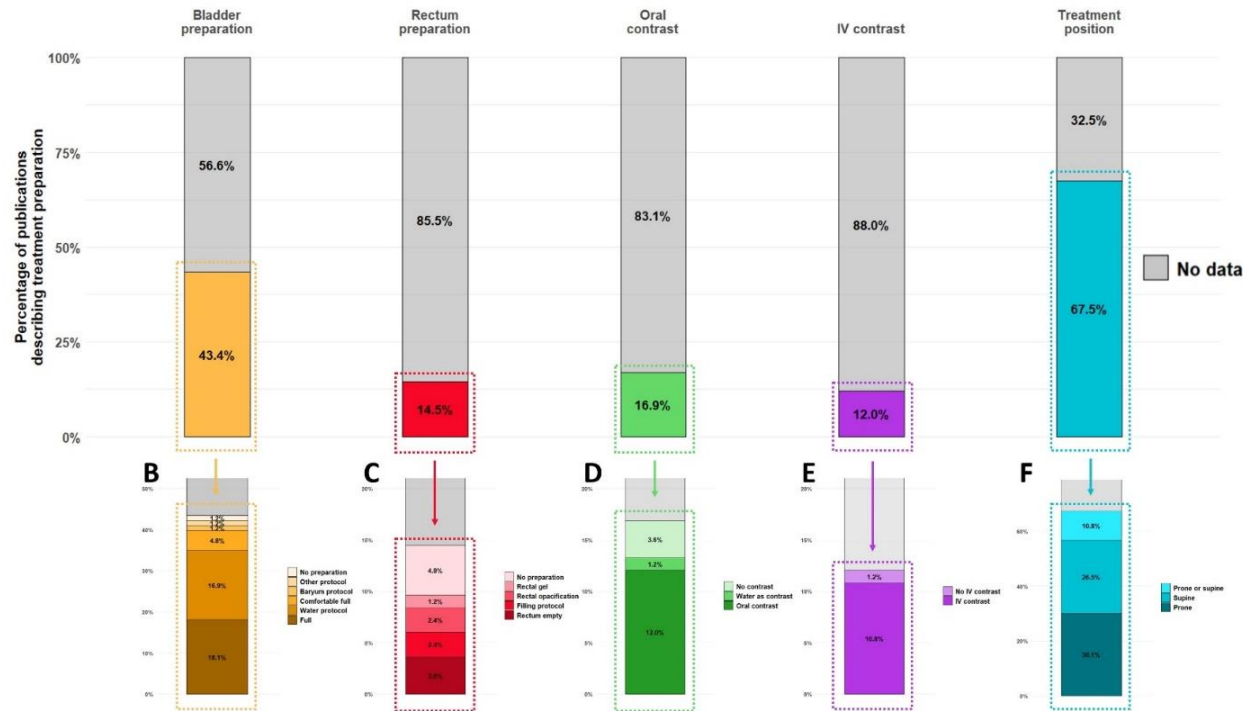


Figure 3.2.2: Radiotherapy treatment preparation parameters (A) across included studies (n = 83). (B) Details of bladder preparation in 36/83 studies, (C) rectum preparation in 12/83 studies, (D) oral preparation in 11/83 studies, (E) IV contrast use in 10/83 studies, and (F) treatment position in 56/83 studies reporting the corresponding parameter. IV: Intravenous.

#### 3.2.3.1.4. RT Boost volumes: marked heterogeneity across studies, from tumour-only to inclusion of total mesorectum and presacral space.

As with RT preparation, substantial inter-study variability was observed in the boost volume definition. In 10.8% (9/83) of studies, the boost target was defined merely as the “primary tumour” with no further details, making it impossible to distinguish between gross tumour volume (GTV), CTV, and PTV per ICRU standards [410]. These studies were excluded from the subsequent analysis.

Among the remaining studies, 73.0% (54/74) reported using multimodality imaging (

Figure 3.2.3A). The imaging and clinical modalities employed for GTV delineation were, in descending order, MRI (85.2%, 46/54), positron emission tomography (PET-CT, 44.4%, 24/54), digital rectal examination (27.8%, 15/54), endoscopy (colonoscopy/rectoscopy, 24.1%, 13/54), and endorectal ultrasonography (20.4%, 11/54,

Figure 3.2.3B).

CTV definition was missing in 4.1% (3/74) of publications. When described, 35.2% (25/71) applied no margin to the GTV, 26.8% (19/71) used numerical margins, 16.9% (12/71) applied purely anatomical margins, and 21.1% (15/71) combined both approaches. Numerical margins (including publications also using anatomic margins) were  $\leq 10\text{mm}$  in 8 studies [307, 335, 342, 343, 348, 355, 387, 390],  $> 10$  and  $\leq 20\text{mm}$  in 18 studies [332, 337,

338, 349, 353, 354, 360, 361, 363, 364, 368, 369, 371, 383-385, 391, 411], and > 20 mm in 4 studies [336, 365, 382, 412]; four used anisotropic numerical margins [373, 374, 376, 388]. Anatomic margins included: circumferential involved rectum wall in 5 papers [103, 113, 338-340], involved mesorectum in 15 papers [336, 337, 342, 353, 354, 364, 366, 368, 375, 381, 386, 390, 399, 408, 412], entire presacral space in 2 papers [371, 382], or other anatomical definitions in 6 papers [307, 372, 373, 376, 400] (

Figure 3.2.3C).

Empirically defined internal target volume margins were reported by 3 teams [339, 342, 348].

PTV margins were described in 75.7% (56/74) of publications, and among them, 80.4% (45/56) used isotropic expansion (

Figure 3.2.3D). Two teams incorporated anatomic boundaries into their PTV definition [103, 113].

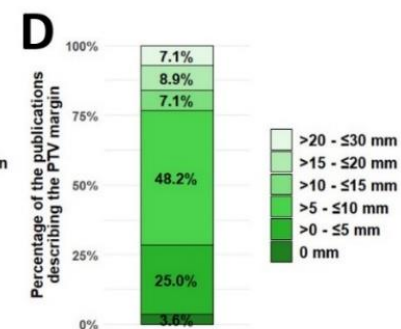
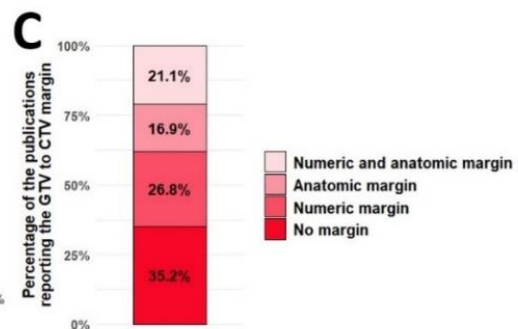
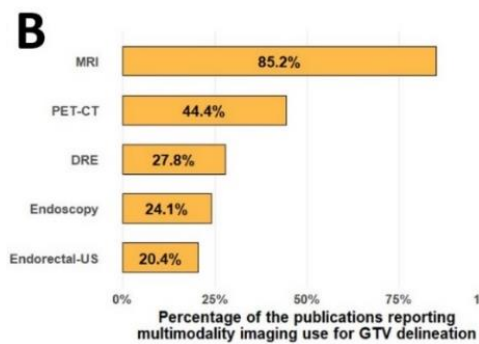
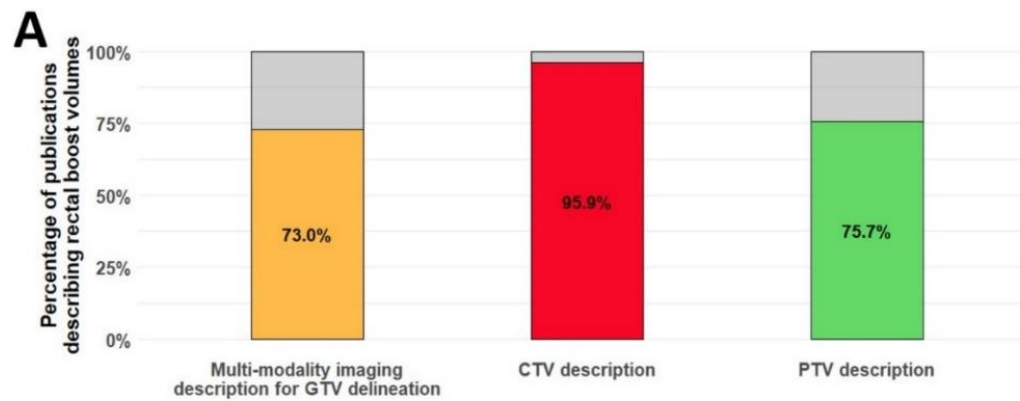


*Figure 3.2.3: RT boost volume definitions and delineation methods. (A) Proportion of studies reporting the GTV, CTV and PTV description among the 74 studies describing their boost volumes. (B) Multi-modality imaging use in publications describing GTV delineation (54/74 studies). (C) GTV to CTV margin strategies among the studies reporting CTV definition (71/74). (D) PTV margin sizes among publications describing PTV boost (56/74). If margins were anisotropic, the largest value was reported. Two studies additionally incorporated anatomical boundaries alongside numeric margins. CTV: Clinical target volume, DRE: Digital rectal examination, GTV: Gross tumour volume, MRI: Magnetic resonance imaging, PET-CT: Positron emission tomography - computed tomography, PTV: Planning target volume, US: Ultrasonography.*



3.2.3.1.5. RT dose: rectal boost biologically effective dose ranges from 56.9 to 103.2 Gy.

All doses prescriptions were converted into biologically effective dose. Equivalencies for common clinical dose are provided in Table A7.1.

Across the 83 included publications, 117 distinct cohorts were identified based on dose prescription. The median biologically effective dose for the elective pelvic volume was 61.0 Gy (range: 49.7 - 69.8 Gy), while the median biologically effective dose for the boost volume was 70.5 Gy (range: 56.9 - 103.2 Gy, Figure 3.2.4A). The median number of fractions was 25 (range: 5 – 38) for the elective pelvic volume and 3 (range: 1 – 14) for sequential boosts (Figure 3.2.4B).

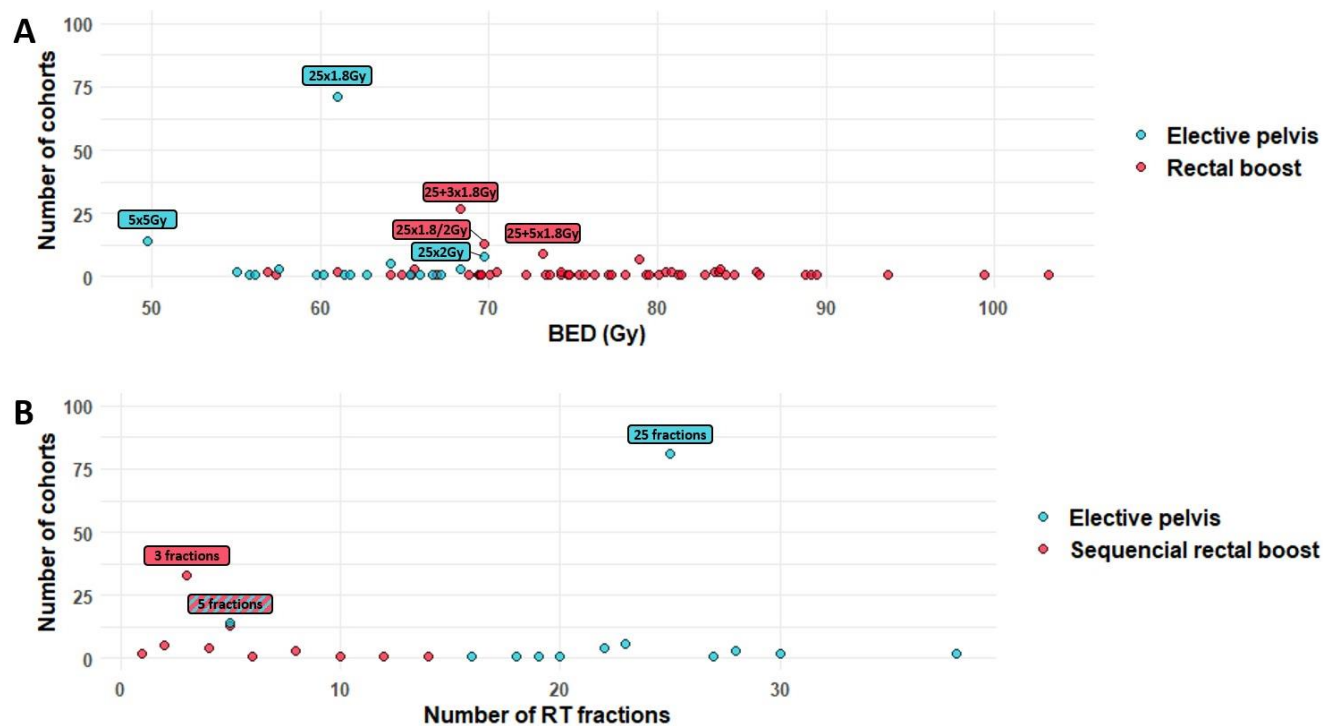


Figure 3.2.4: (A) Prescribed RT dose in the 117 different cohorts identified from the 83 included publications, for both the elective pelvic volume and the rectal boost volume. (B) Number of fractions delivered to the elective pelvic volume and the boost volume when given sequentially. BED: Biologically effective dose, RT: Radiotherapy.

3.2.3.1.6. Chemotherapy: concomitant fluoropyrimidine is standard; induction and/or consolidation chemotherapy is still not frequent.

Concomitant chemotherapy was not reported in 15.7% (13/83) of studies (

Figure 3.2.5A). Among the remaining 70 studies, multiple regimens were described, resulting in 97 different cohorts. Of these: 8.2% (8/97) received no chemotherapy, 74.2% (72/97) received single-agent, and 17.5% (17/97) received doublet. The most frequently used single-agents were capecitabine (44.3%, 43/97), and 5-fluorouracil (27.8%, 27/97). The most common doublet was capecitabine + oxaliplatin (7.2%, 7/97,

Figure 3.2.5B). Less frequent regimens included raltitrexed [307], UFT [339], capecitabine + anakinra [359], capecitabine + bevacizumab [361], irinotecan + s-1 [375], and UFT + oxaliplatin [398].

Data on induction and/or consolidation chemotherapy were unavailable in 16.9% (14/83,

Figure 3.2.5A) of studies. Among the studies that did report this information: 79.7% (55/69) used no additional systemic treatment, 7.2% (5/69) reported induction chemotherapy [331, 358, 361, 385, 394], 7.2% (5/69) reported consolidation chemotherapy [342, 377, 404, 405, 411], 2.9% (2/69) reported either induction or consolidation chemotherapy [103, 351], and 2.9% (2/69) administered both induction and consolidation chemotherapy [378, 398] (

Figure 3.2.5C). Among the 19 reported induction and/or consolidation regimens: 36.8% were single-agent (mostly 5-fluorouracile [342, 378, 385, 411]), and 57.9% were doublets, typically fluoropyrimidine + oxaliplatin [103, 331, 377, 378, 385, 394, 405] (

Figure 3.2.5D). The regimen was not described in 1 study [351]. The use of induction and/or consolidation chemotherapy appears to be a relatively recent development, as reflected by a median publication year of 2019 for studies reporting such regimens. Only five studies implementing these strategies were published before 2017 [331, 361, 385, 398, 411].

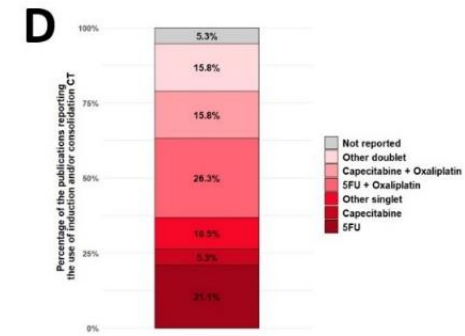
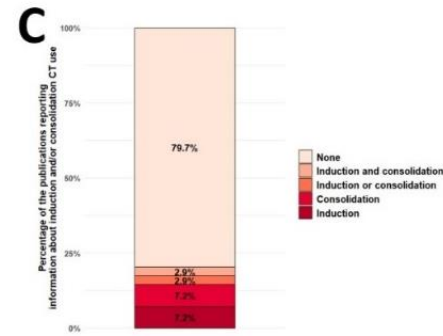
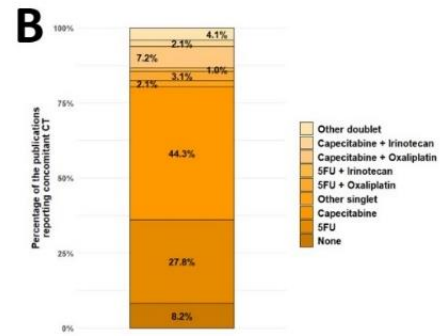
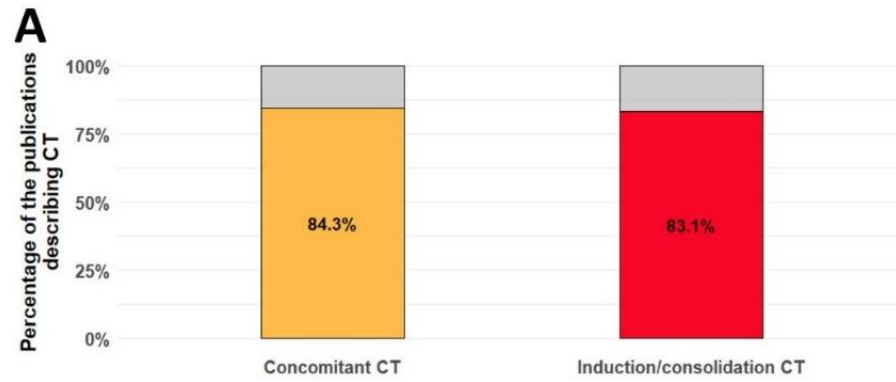


Figure 3.2.5: (A) Proportion of studies reporting data on concomitant and on induction and/or consolidation chemotherapy. (B) Distribution of concomitant chemotherapy regimens across the 97 cohorts identified among the studies that reported this information (70/83). (C) Chemotherapy sequencing (induction and/or consolidation) in the studies reporting such data (69/83). (D) Regimens used in the 19 cohorts reporting induction and/or consolidation chemotherapy. 5FU: 5-fluorouracile, CT: Chemotherapy.

#### 3.2.3.1.7. Treatment strategy: the «Watch and Wait» approach is increasingly adopted.

The post-RT treatment strategy was planned surgery in 63.9% (53/83) and «Watch and Wait» in 15.7% (13/83) publications, while 20.5% (17/83) did not report their approach. The median publication year was 2022 for «Watch and Wait» vs. 2017 for planned surgery ( $p < 0.001$ ).

Among the planned surgery publications, surgical delay was <9 weeks in 43.4% (23/53), >9 weeks in 7.5% (4/53), overlapping both categories in 41.5% (22/53), and not reported in 7.5% (4/53).

In «Watch and Wait» publications, clinical response assessment involved MRI in 76.9% (10/13), endoscopy in 69.2% (9/13), digital rectal examination in 46.2% (6/13), CT in 23.1% (3/13), PET-CT in 7.7% (1/13) [373], and endorectal ultrasound in 7.7% (1/13). No information was provided in 2 publications [340, 358]. The timing of clinical complete response assessment ranged from 6 to 24 weeks. It was not specified in 3 publications [358, 373, 380] (Table A7.7). The frequency of follow-up evaluations was missing in 5 publications [334, 340, 358, 380, 387]. This frequency differed among teams, but generally followed a schedule of every 3 months for 2 years, then every 6 – 12 months for the subsequent 3 years [103, 121, 339, 347, 359, 360, 373, 397].

### 3.2.3.2. *Meta-analysis results*

Of the 141 initially eligible studies (including 212 different cohorts), 63 were excluded following quality assessment (Table A7.9 and Figure A7.14), leaving 78 publications with 97 analysable cohorts. The most frequent reasons for exclusion were scoring “C” for “Correct terminology” (30.7%, 65/212) and “Complete RT description” (20.8%, 44/212) criteria. Conversely, “Cohort selection” and “Outcome assessment” were rated “A” in 99.5% (211/212) and 88.2% (187/212) of cases, respectively, reflecting a good confidence in the meta-analysis results based on these criteria. Among the 97 retained cohorts, 87 reported pathological complete response rates ( $n = 7125$  patients, Table A7.10), and 10 reported clinical complete response under a «Watch and Wait» strategy ( $n = 927$  patients, Table A7.11).

Because the primary objective was to investigate subgroup-specific associations, global pooled meta-analyses were not reported. The following factors were significantly associated with higher pathological complete response rates: using IMRT/VMAT instead of 3D-RT ( $p = 0.007$ ), giving a simultaneous integrated boost rather than a sequential boost ( $p = 0.020$ ), delivering a boost dose  $>74$  Gy in biologically effective dose (equivalent to  $>53$  Gy in 2 Gy fractions,  $p = 0.035$ ), and using a combination of induction and consolidation chemotherapy ( $p = 0.023$ ). Publication year ( $p = 0.14$ ), CTV ( $p = 0.75$ ) and PTV ( $p = 0.10$ ) definitions, concomitant chemotherapy ( $p = 0.10$ ), and surgical delay ( $p = 0.86$ ) were not associated with pathological complete response rate (Figure 3.2.6 and Figure A7.15 for detailed results).



Meta-regression confirmed that the boost biologically effective dose dose was a significant continuous predictor of pathological complete response ( $p < 0.001$ , Appendix 7.3). Between-study heterogeneity was substantial, with  $I^2$  values ranging from 55% to 81% across subgroups (Table A7.12). Significant publication bias was detected (Peters' test  $p$ -value = 0.013, Figure A7.16).

Results regarding clinical complete response in «Watch and Wait» and local recurrence rate in planned surgery and «Watch and Wait» studies are described in Appendix 7.2.

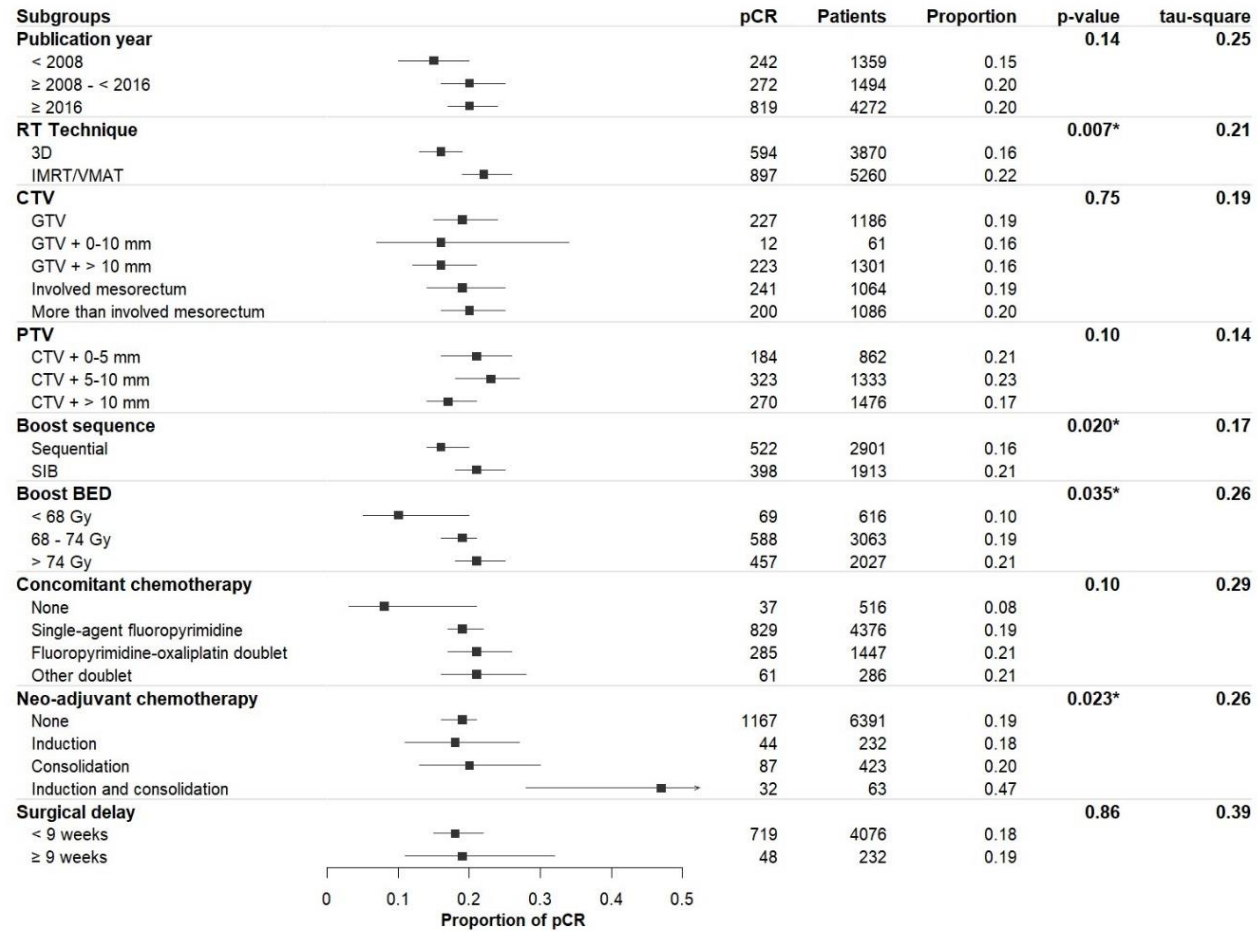


Figure 3.2.6: Subgroup meta-analysis of planned surgery studies reporting pathological complete response rates. The “pCR” column indicates the number of pathological complete response events within each subgroup. The “Patients” column reports the total number of patients included in each subgroup across the pooled studies. The “Proportion” column is the estimated pathological complete response rate. The “p-value” corresponds to the statistical comparison between subgroups, calculated using a mixed-effects model and the Q-test. The “tau-square” column refers to the between-study heterogeneity variance, assessed using the maximum likelihood estimator. \*Significant p-value (<0.05). 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, pCR: Pathologic complete response, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

### **3.2.4. Discussion**

This systematic review highlights a substantial heterogeneity in how external beam radiotherapy rectal boosts are implemented across institutions worldwide. This variability is exemplified by the wide range of CTV definitions (

Figure 3.2.3C), the 52 different dose prescriptions (Figure 3.2.4A), and the 13 different concomitant chemotherapy regimens (

Figure 3.2.5B). Such diversity underscores the need for international guidelines to standardise RT rectal boost protocols in LARC. Our systematic review suggests that, in current practice, most centres use modern RT techniques (IMRT/VMAT) to deliver either a sequential or simultaneous boost, typically in supine position. Bladder filling protocols are frequently applied, whereas rectal preparation protocols remain uncommon. Boost CTV delineation is highly inconsistent, varying from GTV alone to the entire mesorectum. The median biologically effective dose of the boost delivered to the primary tumour (70.5Gy) remains modest, corresponding to approximately 50-54Gy in 25 fractions, a standard prescription. Concomitant radiochemotherapy is common, most often based on fluoropyrimidines. However, the use of induction and/or consolidation chemotherapy remains limited to more recent studies. Meta-analysis results demonstrate that the use of modern RT techniques (IMRT/VMAT) compared to 3D, a simultaneous integrated boost compared to a sequential boost, and higher biologically effective dose doses (>74 Gy, equivalent to >53 Gy in 2 Gy fractions) are all significantly associated with improved pathological

complete response rates. Due to the limited number of studies reporting outcomes in the «Watch and Wait» setting, no conclusion can be drawn regarding clinical complete response. In the following sections, we summarise the main findings in light of the current literature and provide technical insights to support the implementation of rectal boost external-beam RT in LARC patients.

In rectum cancer, only a limited number of studies have compared 3D with IMRT/VMAT. Dosimetric studies have shown that IMRT provides superior target coverage conformity and improved sparing of organs at risk (OARs) compared to 3D [406, 413]. However, retrospective comparative studies, typically involving small cohorts, failed to demonstrate any difference in response and survival outcomes, although they suggest a reduction in radiation-induced toxicities with IMRT/VMAT [388, 414]. Our descriptive analysis confirms that these conformal techniques are now widely established in clinical practice. Moreover, the meta-analysis supports the superiority of IMRT/VMAT over 3D when delivering an external-beam RT boost to the primary tumour. This analysis does not aim to provide conclusions regarding alternative rectal boost modalities such as contact x-ray brachytherapy. Prospective trials are required to validate whether the observed improvements in pathological complete response rates can translate into higher clinical complete response and organ preservation rates, as well as improved OAR sparing—outcomes that were beyond the scope of the present work.

By shortening the overall treatment time and increasing the dose per fraction, a simultaneous integrated boost may theoretically limit tumour cell repopulation and increase tumour control, which has never been confirmed in other cancer type trials or in rectal cancer retrospective publications [342, 373, 388, 415-420]. However, dosimetric and toxicity benefits have been highlighted for simultaneous boost for rectal cancers as well as in other cancers [342, 388, 415-417]. Additionally, simultaneous integrated boost also offers practical benefits, including reduced number of treatment sessions, with potential economic and patient comfort advantages [421]. While our descriptive analysis indicates that sequential and simultaneous boosts are used with similar frequency, our meta-analysis shows that delivering the boost simultaneously with pelvic irradiation is significantly associated with higher pathological complete response rates compared to sequential approaches. These findings support the inclusion of simultaneous integrated boost in future recommendations to encourage its broader adoption in clinical practice.

Rectum and bladder preparations are important for the successful completion of RT in rectal cancer, serving 2 main objectives: (1) limiting toxicity by displacing the small bowel and other pelvic OARs out of the high-dose region, and (2) ensuring interfractional anatomical reproducibility, thereby avoiding dosimetric deviation from the scheduled RT plan [422-424]. In practice, although strict rectal preparation protocols are feasible, they are subject to random daily variations and patient compliance issues. These uncertainties must be accounted for in PTV margins [424, 425]. Technologies such as CBCT and MRI-guided online-adaptive RT are emerging

as effective solutions to manage daily anatomical variability and are particularly relevant in pelvic RT, where interfraction organ motion is considerable [426, 427]. Several teams have already implemented online-adaptive RT for rectal cancers, showing promising findings regarding PTV reduction and dosimetric results [194, 276, 428]. In our systematic review, most studies reported using a full bladder protocol, while rectal preparation was less consistently addressed. Regarding treatment position, both prone and supine offer advantages: prone position can reduce the small bowel dose, while supine position improves reproducibility and patient comfort. With advances in RT delivery and image-guidance, the differences between these positions are becoming less relevant [311, 429, 430]. Our analysis revealed that both were commonly used, with a trend toward increased use of the supine position in recent years.

The greatest interstudy variability was observed in the boost volume definitions (GTV, CTV, PTV). Furthermore, several studies did not use the standardised denomination recommended by the International Commission on Radiation Units and Measurements (ICRU) report No. 83 [431], leaving therefore a risk of inaccurate results. Current guidelines recommend the integration of digital rectal examination, endoscopy and MRI to guide the delineation in rectal cancer. Particularly, high-resolution T2-weighted MRI allow direct visualisation and accurate delineation of the tumour and its relationship to the mesorectal fascia [432]. While the use of PET-CT for GTV delineation is not recommended, preliminary studies suggest it may reduce interobserver delineation variability and GTV size [400, 433-435]. Diffusion-weighted MRI also appears promising, with data suggesting improved

interoperator reproducibility and further GTV reduction when compared to PET-CT [436]. In our descriptive analysis, MRI was the most frequently reported imaging modality for GTV definition and should be recommended for this purpose. The lack of consistent reporting regarding volume definition and imaging modalities underscores once again the need for clear guidelines on rectal boost delineation, although such recommendations already exist for the elective CTV (pelvic lymph nodes) definition [161]. This gap was reflected in our findings: some teams applied numerical margins around the GTV, others used anatomical landmarks, and some combined both. The resulting CTV volumes ranged widely, from the primary tumour alone to the entire mesorectum or even presacral space. Importantly, the meta-analysis results showed that the CTV definition did not significantly impact CR rate or local recurrence rate. These results, aligned with the objective of non-operative management and limitation of total irradiated volume, support the consideration of no additional GTV to CTV margin, especially given that the whole rectum and mesorectum are already irradiated as part of the prophylactic pelvic RT [161]. Future trials should investigate whether limiting the boost CTV could safely validate those principles. PTV margins were most often defined empirically and isotropically, though not always supported by experimental and clinical evidence. Anisotropic PTV expansions have also been used to account for the variability of rectal motion in the 3 dimensions, which is greater in the antero-posterior and supero-inferior axes than in the left-right axis [296, 302, 303]. Other groups have suggested more individualised PTV definitions incorporating setup errors, delineation errors, rectal motion, gender, and



tumour location [276, 311, 390, 437]. The meta-analysis found no impact of the PTV boost definition on RT outcomes, suggesting that margin definition should be adapted to each institution's protocols (image-guided RT, adaptive RT, RT preparation, etc.).

Two neo-adjuvant RT regimens are currently recommended for LARC: (1) short-course RT (25 Gy in 5 fractions), and (2) long-course RT (45-50 Gy in 25 fractions with a boost up to 54-56 Gy) [94]. A meta-analysis by Burbach et al. (2014) showed that escalating the dose above 60Gy (EQD2) was associated with high pathological complete response, good resectability and acceptable toxicity [163]. Our systematic review revealed that the clinical practice often diverges from standard RT fractionations, with more exotic schemes. We converted all boost doses in biologically effective dose and categorised them into 3 categories: (1) biologically effective dose < 68 Gy, mainly short-course regimens; (2) biologically effective dose 68 – 74 Gy, standard long-course regimens; and (3) biologically effective dose > 74 Gy, dose-escalation regimens. Both subgroup meta-analysis and meta-regression demonstrated a positive correlation between dose escalation and pathological complete response, although the absolute level of escalation remains modest (e.g. 74Gy biologically effective dose  $\approx 25 \times 2.09\text{Gy}$ ). In contrast, no association was found in «Watch and Wait» studies or for local recurrence rate, likely due to smaller cohorts in the «Watch and Wait» analysis and heterogenous follow-up for local recurrence rate. Nevertheless, these findings support further exploration of rectal boost dose escalation, provided toxicity remains acceptable, which was not assessed here.

Single-agent fluoropyrimidine is the standard during long-course RT. Doublet regimens demonstrated a higher toxicity without consistent oncological benefit [108, 285, 438-440]. In our analysis, over 70% of cohorts used 5-fluorouracile or capecitabine alone, and doublet regimens did not improve outcomes. TNT, validated in recent phase III trials (PRODIGE-23 and RAPIDO), remains a new paradigm, with both studies published in 2021 [102, 115]. Reflecting this, only 14 of the 83 publications included in our descriptive analysis reported the use of induction and/or consolidation chemotherapy. In the meta-analysis, only the combination of induction and consolidation chemotherapy showed a significant pathological complete response benefit. However, this was based on one retrospective and one small phase I study, deserving confirmation in prospective studies [378, 398]. Unexpectedly, an increased local recurrence rate was observed in «Watch and Wait» cohorts using induction or consolidation chemotherapy, coming exclusively from the “OPRA” study [103]. The proportion of patients managed with a non-operative approach in the “OPRA” study was higher than other «Watch and Wait» studies—except the study of Boeke et al., 2022, which included only five patients with limited follow-up [103, 121, 164, 347, 380]. This study likely applied less stringent criteria for non-operative management, therefore leading to the inclusion of patients with residual disease despite initial clinical complete response after TNT and a higher risk of local recurrence. Also, these results could be due to the interstudy difference in follow-up. These results should be taken cautiously, and no conclusions should be drawn over those of randomised phase III trials, without future robust prospective evaluations. Finally, most studies

evaluating induction and/or consolidation chemotherapy focused on survival endpoints, not local control, limiting their integration in this meta-analysis.

Planned surgery remains the standard approach, with a 6 – 8-week delay between neo-adjuvant treatment and surgery being associated with higher pathological complete response rates [94, 441]. However, the «Watch and Wait» strategy is gaining interest, aiming to preserve organ and function and the avoidance of a surgery in patients achieving clinical complete response, thereby improving quality of life [287]. With TNT, «Watch and Wait» patients can reach over 50% of three-year total mesorectal excision-free rates [103]. The recommended clinical evaluation after neo-adjuvant therapy includes digital rectal examination, rectoscopy, and MRI, the modalities that are most frequently reported in our descriptive analysis. In planned surgery cohorts, no significant pathological complete response or local recurrence rate difference was observed between surgical delays < 9 weeks and  $\geq 9$  weeks. However, these results are limited by the small number of studies in the second category.

Regarding limitations of this study, to provide the most up-to-date information in the descriptive analysis, we included only the most recent publication from each team, which may not always reflect the most detailed descriptions of RT boost techniques. Also, although our research algorithm was chosen to be as wide-ranging as possible, some relevant publications may have been overlooked due to the extensive literature on rectal radiotherapy. A considerable proportion of studies were excluded based on

the quality evaluation: 27.8% (32/115) in the descriptive analysis and 44.7% (63/141) in the meta-analysis, due to stringent criteria. This ensure high-quality data but may have led to the exclusion of studies that could have significantly influenced the meta-analysis results (e.g., studies with large cohorts) or that had strong methodological designs (e.g., prospective studies). The meta-analysis aimed to identify RT parameters associated with increased complete response rates, potentially guiding strategies to expand eligibility for «Watch and Wait». However, the «Watch and Wait» literature remains limited, with short follow-up and small cohorts, contributing to inconclusive findings, particularly regarding induction and/or consolidation chemotherapy. To limit this bias, only studies with at least 12 months of follow-up were included. In contrast, planned surgery publications were more numerous, providing valuable findings on RT practices to optimise pathological complete response. Still, although pathological complete response is associated with better long-term oncological outcomes, its translation into clinical complete response for a «Watch and Wait» strategy remains uncertain [321, 442, 443]. Each factor in the meta-analysis was analysed independently, which may be misleading. For instance, IMRT/VMAT use increased significantly over time ( $p < 0.001$ ), as did the use of simultaneous boosts ( $p = 0.09$ ) and the use of induction and/or consolidation chemotherapy. These co-evolutions undermine the assumption of variable independence. Since all the evaluated factors were inconstantly reported in a high proportion of the studies, adjusting the results for dependency would require extensive imputation of missing values, which could distort representativeness. Additionally, we focused

exclusively on RT parameters, whereas tumour, biological, and socio-economic factors also influence pathological/clinical complete response [441, 444-448]. Important tumour-related predictors include the tumour size [441, 444, 446, 448], the T stage [441, 444, 446], the N-stage [441], the differentiation grade [441, 446], the circumferential invasion [446], the macroscopic ulceration [446]. In some studies, RT-related parameters (RT technique, RT dose, surgical delay) were not associated with pathological complete response [446, 448]. Biological parameters such as low carcinoembryonic antigen, neutrophil-to-lymphocyte ratio, and albumin levels, or high haemoglobin and lymphocyte counts, have also been associated with higher pathological complete response probability, sometimes more than demographic features including age, gender, and body mass index [444, 446]. In addition, advanced methods like machine learning and genomic profiling are being explored to predict pathological complete response in LARC, further underlining the relevance of this outcome in LARC patients. Therefore, RT parameters should not be viewed as the sole determinants of treatment response, and potential confounding factors must be acknowledged. Regarding local recurrence rate, given the variable follow-up durations of the included publications, results should be interpreted cautiously (Appendix 7.2). Finally, interstudy heterogeneity was high, particularly for pathological complete response and clinical complete response results, calling for cautious interpretation. Significant publication bias was also detected in planned surgery studies but not in «Watch and Wait» studies. However, Peter's test for publication bias analysis lacks

power when the cohort number is lower than 10, as was the case for «Watch and Wait» publications [449].

### **3.2.5. Conclusion**

This systematic review highlights the important variability in how external-beam RT boosts to the primary rectal tumour are delivered in patients with LARC worldwide. The meta-analysis provides insights into RT technique, the RT preparation, the RT boost delineation, the RT dose, the chemotherapy, and the treatment strategy. It demonstrates that higher pathological complete response rates are significantly associated with the use of IMRT/VMAT over 3D, simultaneous rather than sequential boost delivery, RT dose escalation, and the combination of induction and consolidation chemotherapy. However, these results should be interpreted cautiously, as the analysis assumes independence between variables, despite known temporal and clinical interdependencies, and considers only technical treatment parameters, omitting key clinical factors correlated with pathological complete response such as the tumour stage or size. The positive correlation between pathological complete response and the RT dose was confirmed further by meta-regression, reinforcing the potential benefits of RT dose escalation for organ preservation strategies. Nonetheless, literature on this «Watch and Wait» approach remains limited. Therefore, no definitive conclusions can be drawn from this meta-analysis regarding RT techniques that may improve clinical complete response in «Watch and Wait» settings.

In conclusion, these results underscore the urgent need for clear international guidelines on rectal boost RT. Such recommendations would help standardise clinical practice, improve comparability across future studies, and support the development of non-operative management strategies. Ultimately, this would lead to enhancement of treatment effectiveness and quality of life for patients.

### **3.3. Immune-inducing radiotherapy boost combined with immunotherapy in total neoadjuvant treatment for locally advanced MSS rectal cancer**

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#### **Highlights**

- Non-operative management after total neoadjuvant therapy is an option in locally advanced rectal cancer with clinical complete response
- Delaying tumour-draining lymph node radiotherapy (RT) from primary tumour RT may restore RT-immunotherapy synergy
- Induction immunostimulating RT boost and immunotherapy could increase non-operative management



- Optimisation of a clinical protocol is required before evaluating safety and efficacy of this approach

## **Abstract**

In microsatellite-stable (MSS) locally advanced rectal cancer, non-operative management may be offered to patients who achieve a clinical complete response after total neoadjuvant therapy, thereby avoiding total mesorectal excision. Although immune checkpoint inhibitors have been evaluated in this setting, outcomes remain suboptimal, particularly when compared with microsatellite instability-high tumours. Consequently, novel total neoadjuvant therapy strategies are required to improve clinical complete response rates and increase the proportion of patients eligible for non-operative management.

We propose here a clinical protocol designed to evaluate an experimental total neoadjuvant treatment strategy for patients with MSS locally advanced rectal cancer. This protocol includes an initial hypofractionated radiotherapy (RT) boost to the primary tumour with delayed long-course chemoradiotherapy to tumour-draining lymph nodes, immunotherapy, and chemotherapy. However, defining the optimal integration and parameterization of these treatment modalities remains challenging.

For the initial rectal tumour RT, hypofractionated regimen can promote neoantigen release and immune activation. At the same time, the irradiated volume should be carefully limited to avoid unnecessary tumour-draining lymph node irradiation. Heterogeneous tumour irradiation may represent a suitable strategy to reach these two objectives. Regarding immunotherapy, several agents may be considered, ranging from well-established anti-PD-(L)1 to less extensively studied drugs. Treatment sequencing is also critical,

as concomitant or immunotherapy-first administration has been shown to be less effective than radiotherapy-first strategies. Chemotherapy, particularly oxaliplatin-based regimens commonly used in locally advanced rectal cancer, may further contribute to immune activation. Chemotherapy can potentially synergise with immunotherapy, especially when immunotherapy is given first.

Overall, this work provides a framework for discussion and can be viewed as a perspective for the thesis as a whole, integrating the RT techniques developed, the experience acquired in analysing RT-induced immune responses, and recent advances reported by other research groups.

### **3.3.1. Background and objectives in locally advanced rectal cancer**

Rectal cancer is a major public health issue, with 730,000 new cases and 343,000 deaths reported annually [1]. Managing locally advanced rectal cancer (T3/4 and/or N+ disease) is complex and presents multiple challenges [94].

A primary objective is to achieve local control. Total mesorectal excision provides superior local control compared with tumour excision alone [97]. Adding neoadjuvant radiotherapy (RT), either as short-course RT (5 fractions) or long-course chemoradiotherapy (25 fractions), further improves local outcomes [98, 100]. To achieve greater local control, escalating the RT dose to the primary tumour is a potential option [162]. Endorectal brachytherapy boosts can achieve high clinical complete response rates in early LARC, enabling a non-operative strategy. In the OPERA trial, adding an endorectal brachytherapy boost of three 30 Gy fractions after long-course chemoradiotherapy led to a 3-year organ preservation rate of 81%, significantly higher than long-course chemoradiotherapy alone (59%;  $p = 0.003$ ) [121]. However, the benefit of an external-beam RT boost remains more controversial [121, 163, 164, 276].

A second objective is to reduce metastatic spread, which significantly impacts overall survival [125]. Recently, total neoadjuvant therapy (TNT), combining neoadjuvant chemotherapy and RT, has demonstrated improved metastatic control compared with long-course chemoradiotherapy in two phase III trials: RAPIDO and PRODIGE-23 [115, 160]. The RAPIDO regimen

consists of short course RT followed by consolidation chemotherapy (5-fluorouracil/capecitabine and oxaliplatin). While this approach achieved its primary endpoint of reducing the 3-year disease-related treatment failure with a reduction of distant metastases, long-term data reveal decreased local control compared with long-course chemoradiotherapy [116, 160]. In PRODIGE-23, patients receiving TNT with induction chemotherapy (5-fluorouracil, leucovorin, oxaliplatin, and irinotecan; FOLFIRINOX) followed by long-course chemoradiotherapy showed higher 3-year disease-free survival and improved overall survival on long-term follow-up compared to long-course chemoradiotherapy alone [104, 115].

A third objective is the preservation of patients' quality of life. Non-operative management is an emerging strategy for patients achieving a clinical complete response after total neoadjuvant treatment [94, 103, 287]. In localised and early locally advanced rectal cancer (cT1–3b N0 tumours,  $\leq 40$  mm), the STAR-TREC trial recently demonstrated that non-operative management using long-course chemoradiotherapy (with irradiation limited to the mesorectum) can achieve up to 80% organ preservation at 1 year [450]. In contrast, for larger tumours, higher Tumor-Node-Metastasis (TNM) stages or greater circumferential involvement, complete response rates after neoadjuvant therapy are significantly lower [441, 446]. Based on institutional experience (unpublished) and current literature in locally advanced rectal cancer, although clinical complete response is observed in approximately 50% of cases, tumour regrowth occurs in about 20%, resulting in an overall organ preservation rate of around 30% [103, 287]. Importantly, in this population, non-operative management does not appear to

compromise long-term oncological outcomes, including distant recurrence-free and overall survival [451, 452]. Moreover, early response to TNT correlates with improved oncological outcomes in LARC, supporting treatment intensification [451, 452]. Therefore, increasing the proportion of patients that can avoid total mesorectal excision is a major objective in LARC to preserve their quality of life. Increasing the proportion of patients who can avoid total mesorectal excision is therefore a major objective, given the substantial morbidity of surgery, including faecal incontinence in approximately 60% of cases, major low anterior resection syndrome in nearly 50%, and potential urinary and erectile dysfunction [118-120].

Several strategies are under investigation, including RT dose escalation (already mentioned) and immunotherapy. While immune checkpoint inhibitors induce high and durable response rates in mismatch repair-deficient/high microsatellite instability (dMMR/MSI-H) rectal cancer ( $\approx 5\%$  of CRC), efficacy in microsatellite-stable (MSS) tumours remains limited [74, 81, 123, 151, 152, 453]. A leading hypothesis for these disappointing results is that RT to tumour-draining lymph nodes (TDLNs), although essential for oncological control, impairs RT-immunotherapy synergy [100, 247-249, 252, 253]. Nevertheless, combining RT with immunotherapy remains highly attractive because of the immune stimulation induced by irradiation. RT promotes the release of tumour neoantigens, which are captured and processed by antigen-presenting cells that subsequently migrate to tumour-draining lymph nodes to prime CD8<sup>+</sup> T cells. Activated CD8<sup>+</sup> T cells then infiltrate the tumour microenvironment, where they mediate immunogenic tumour cell death. This mechanism has been shown in multiple preclinical

models to synergise with immune checkpoint inhibition and enhance its therapeutic efficacy [203, 234, 454]. A recent preclinical study showed that delaying TDLN irradiation after primary tumour RT could restore the efficacy of immune checkpoint inhibitors administered prior to tumour irradiation [249]. Simultaneous irradiation of the primary tumour and TDLNs was associated with reduced intratumoral CD8<sup>+</sup> T-cell infiltration, downregulation of the CCR7–CCL19/CCL21 homing axis, and impaired dendritic-cell antigen presentation compared with delayed TDLN RT [249]. This strategy, combined with RT dose escalation to the primary tumour, may therefore be explored within the total neoadjuvant setting in MSS locally advanced rectal cancer to increase clinical complete response rates and ultimately reduce the need for total mesorectal excision.

### **3.3.2. Proposal for a new total neoadjuvant strategy**

The objective of this strategy is to propose a novel total neoadjuvant therapy approach for patients with MSS locally advanced rectal cancer. The proposed strategy integrates: (1) dose-escalated RT to the primary tumour with delayed irradiation of TDLNs, (2) immunotherapy, and (3) neoadjuvant chemotherapy. The aim is to introduce an innovative regimen capable of substantially increasing complete response rates and, consequently, quality of life, while remaining sufficiently close to current standard-of-care treatments to avoid compromising oncological outcomes or increasing toxicity. As the foundation for this experimental design, the PRODIGE-23 total neoadjuvant therapy regimen will be used [104]. This standard

approach consists of six cycles of mFOLFIRINOX (oxaliplatin, leucovorin, irinotecan, and fluorouracil), followed two weeks later by long-course pelvic RT (44 Gy in 2-Gy fractions) with limited dose escalation to the primary tumour (50 Gy in 2-Gy fractions), combined with concomitant oral capecitabine (

Figure 3.3.1) [115].

#### *3.3.2.1. New total neoadjuvant therapy*

In the experimental approach, treatment will begin with two cycles of concomitant immunotherapy and hypofractionated RT delivered exclusively to the primary rectal tumour (3 fractions of 5 Gy). The objective of this first phase is to trigger an immune response that potentiates immunotherapy while preserving TDLN integrity, which is essential for immune priming. This phase will be followed by six cycles of mFOLFIRINOX administered with concomitant immunotherapy. Finally, long-course chemoradiotherapy with capecitabine will be delivered to the pelvis, without further dose escalation to the primary tumour, again combined with concomitant immunotherapy (

Figure 3.3.1). Patients achieving a clinical complete response will be offered a non-operative management strategy, whereas those with incomplete response or based on patient preference will undergo total mesorectal excision 2 (+/- 2) weeks after definitive response assessment. Oncological



follow-up will be conducted according to current guidelines (Table 3.3.1) [94, 285, 455].

Clinical visits will be scheduled before each immunotherapy (+/- chemotherapy) cycle, including blood sampling and toxicity assessment by a medical oncologist. Weekly evaluations will be performed by a radiation oncologist during RT to monitor treatment-related toxicity. Clinical response assessment, including digital rectal examination, rectoscopy with tumour biopsies, and pelvic MRI, will be performed after concurrent immuno-chemotherapy (early response evaluation) and 8 – 12 weeks after completion of long-course chemoradiotherapy (definitive response evaluation). For translational research purposes, tumour tissue and blood samples will be collected at these same time points (Table 3.3.1).

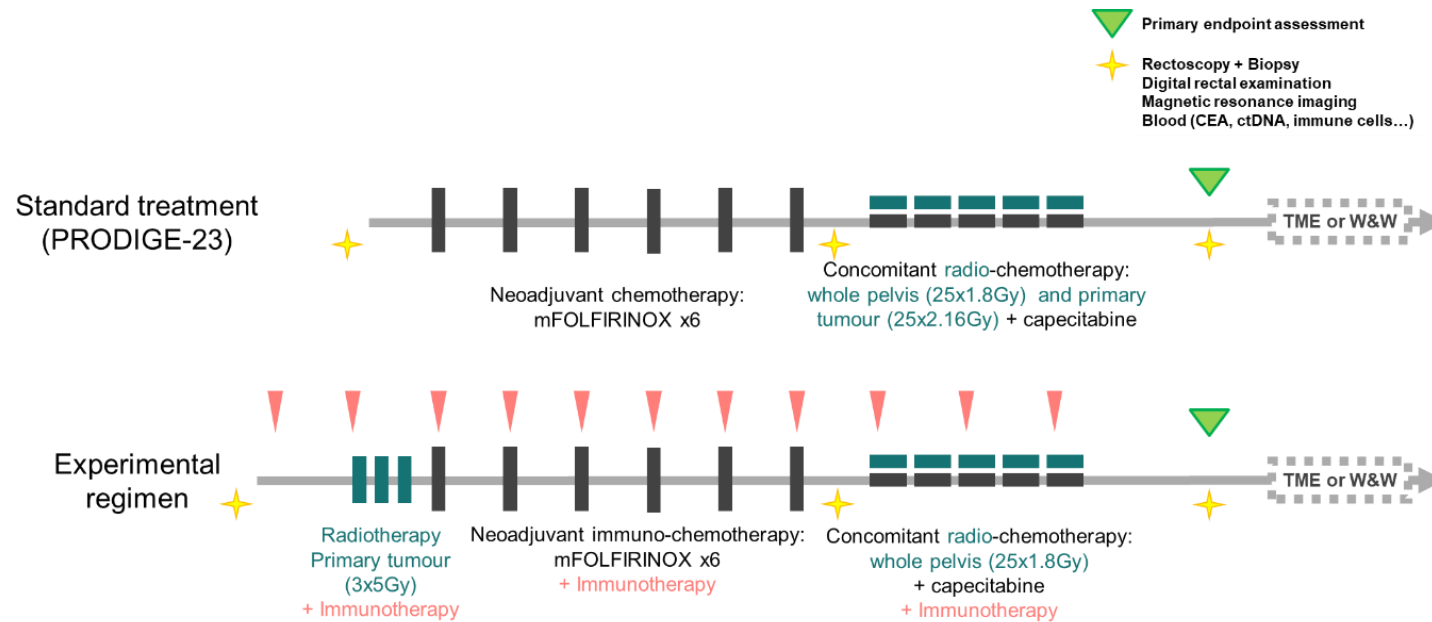


Figure 3.3.1: Proposed study design. The experimental TNT regimen includes an RT boost to the primary tumour to trigger an immune response and enhance the effect of concomitant immunotherapy. This is followed by

*concomitant chemotherapy with mFOLFIRINOX and immunotherapy, then RT to the TDLNs for oncological control combined with continued immunotherapy. Co-primary endpoint assessments (clinical + pathological complete response and safety) are performed after TNT completion (for clinical complete response) and on the surgical specimen (for pathological complete response). Biological sample collection, rectoscopy, and magnetic resonance imaging are carried out at predefined time points. CEA: Carcinoembryonic antigen, ctDNA: Circulating tumour DNA, RT: Radiotherapy, TDLNs: Tumour-draining lymph nodes, TME: Total mesorectal excision, TNT: Total neoadjuvant treatment, W&W: Watch and Wait.*

### *3.3.2.2. Targeted population*

Adult patients with histologically confirmed MSS rectal adenocarcinoma of American Joint Committee on Cancer stage II (T3–4 N0) or stage III (Tx N1–2) and deemed fit for total neoadjuvant therapy will be eligible. Patients with metastatic disease, prior pelvic radiotherapy, autoimmune disease, organ transplantation, or previous exposure to immunotherapy will be excluded.

### *3.3.2.3. Endpoints*

This study will use co-primary endpoints: safety and the combined complete response rate. Safety will be assessed through systematic monitoring of toxicities related to immunotherapy, chemotherapy, and RT throughout total neoadjuvant therapy by both medical and radiation oncologists. Acute toxicity will be evaluated until definitive response assessment after completion of total neoadjuvant therapy, after which long-term oncological follow-up and late toxicity monitoring will be conducted in accordance with current recommendations (Table 3.3.1). Additional safety endpoints will include the proportion of patients completing immune-chemotherapy, the proportion completing long-course immune-chemoradiotherapy, and the feasibility of performing total mesorectal excision (when indicated) within 8 – 16 weeks after total neoadjuvant therapy completion. The combined complete response endpoint will integrate the proportion of patients achieving a clinical complete response (managed with a non-operative strategy) and the proportion achieving a pathological complete response following total mesorectal excision. Pathological complete response will be

determined on surgical specimens obtained after total mesorectal excision (Table 3.3.1).

Secondary endpoints will include survival outcomes (disease-free survival, metastasis-free survival, and overall survival), local control and surgical outcomes (locoregional recurrence rate, local regrowth rate after clinical complete response, total mesorectal excision–free survival, complete resection rate, and total mesorectal excision–related morbidity), as well as quality-of-life outcomes and patient-reported outcome measures (Table 3.3.1).

Biological samples, including tumour tissue and blood, will be collected at three predefined time points: (1) at patient inclusion, (2) at early response evaluation (after completion of immune-chemotherapy), and (3) at definitive response evaluation (8 – 12 weeks after immune-chemoradiotherapy, Table 3.3.1). These samples will be used to characterise the tumour immune microenvironment, to assess its dynamic evolution during treatment, and to correlate biological changes with complete response outcomes. Analyses will be conducted using in-house methodologies and (inter-)institutional platforms (Figure 3.3.2).

Figure 3.3.1 and Table 3.3.1 present a preliminary proposal for the implementation of this study. Given the complexity of the interactions between the different treatment modalities, the following section will examine potential strategies for optimisation of this protocol.

|                                           |           | TNT (23+/-1w)                |                                     |                                      |                                           | Post-TNT strategy (8 - 16w)              |                            | Oncological follow-up (5 years)                       |                                 |
|-------------------------------------------|-----------|------------------------------|-------------------------------------|--------------------------------------|-------------------------------------------|------------------------------------------|----------------------------|-------------------------------------------------------|---------------------------------|
|                                           | Inclusion | Immune priming RT boost (4w) | Induction immuno-chemotherapy (12w) | Early response evaluation (2 +/- 1w) | Long-course immune-chemoradiotherapy (5w) | Definitive response evaluation (8 - 12w) | TME (2+/-2w)               | Years 1-2                                             | Years 3-5                       |
| Inform consent signature                  | X         |                              |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Treatment                                 |           |                              |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Primary tumour RT boost                   |           | X                            |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Long-course RT + Capecitabine             |           |                              |                                     |                                      | X                                         |                                          |                            |                                                       |                                 |
| Immunotherapy                             |           | X                            | X                                   |                                      | X                                         |                                          |                            |                                                       |                                 |
| mFOLFIRINOX                               |           |                              | X                                   |                                      |                                           |                                          |                            |                                                       |                                 |
| Co-primary endpoint evaluation            |           |                              |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Safety                                    |           | X                            | X                                   |                                      | X                                         | X                                        | X                          |                                                       |                                 |
| Clinical + pathological complete response |           |                              |                                     |                                      |                                           | clinical complete response               | clinical complete response |                                                       |                                 |
| Secondary endpoint evaluation             |           |                              |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Oncological secondary endpoints           |           |                              |                                     |                                      |                                           |                                          |                            | 1x/3 months if «Watch and Wait»<br>1x/6 months if TME | 1x/6 months                     |
| Quality of life, PROMs                    | X         |                              |                                     |                                      |                                           | X                                        |                            | 1x/6 months                                           | 1x/6 months                     |
| Oncological work-up                       |           |                              |                                     |                                      |                                           |                                          |                            |                                                       |                                 |
| Carcinoembryonic antigen                  | X         |                              |                                     | X                                    |                                           | X                                        |                            | 1x/3 months                                           | 1x/6 months                     |
| Digital rectal examination                | X         |                              |                                     | X                                    |                                           | X                                        |                            | 1x/3 months if «Watch and Wait»                       | 1x/6 months if «Watch and Wait» |
| Rectoscopy +/- biopsy                     | X         |                              |                                     | X                                    |                                           | X                                        |                            | 1x/3 months if «Watch and Wait»                       | 1x/6 months if «Watch and Wait» |
| MRI                                       | X         |                              |                                     | X                                    |                                           | X                                        |                            | 1x/3 months if «Watch and Wait»                       | 1x/6 months if «Watch and Wait» |

|                           |   |   |   |                              |             |
|---------------------------|---|---|---|------------------------------|-------------|
| Thoraco-abdominal CT-scan |   |   | X | 1x/6 months                  | 1x/6 months |
| Translational research    |   |   |   |                              |             |
| Tumour biopsy             | X | X | X | X if oncological progression |             |
| Blood                     | X | X | X | X if oncological progression |             |

*Table 3.3.1: Proposed schedule of study procedures. CT: Computed tomography, exp: Only for the experimental group. MRI: magnetic resonance imaging, PROMs: Patient-reported outcome measures, RT: Radiotherapy, SIB: Simultaneous integrated RT boost to the primary tumour, TME: Total mesorectal excision, TNT: Total neoadjuvant treatment, w: Week.*

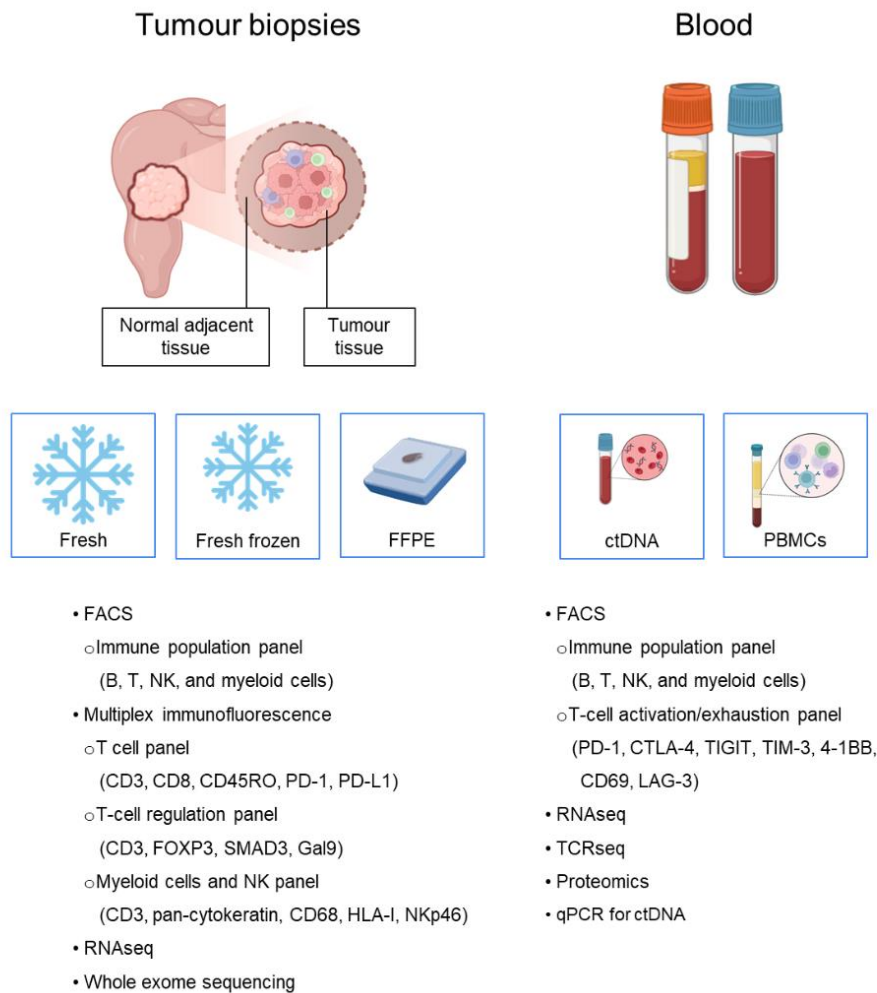


*Figure 3.3.2: Translational analyses for full tumour immune microenvironment characterisation. ctDNA: Circulating tumour DNA, FACS: Fluorescence-activated cell sorting, FFPE: Formalin-fixed paraffin-embedded, MDSC: Myeloid-derived suppressor cells, NK: Natural killer, PBMCs: Peripheral blood mononuclear cells, qPCR: Quantitative polymerase chain reaction.*



### **3.3.3. Radiotherapy considerations**

This protocol aims to translate into clinical practice preclinical findings showing that delayed irradiation of tumour-draining lymph nodes (TDLNs) restores immunotherapy efficacy in order to improve oncological outcomes for patients with a MSS locally advanced rectal cancer [249]. In this model, compared with concurrent primary tumour–TDLN irradiation combined with anti-CTLA-4 therapy, mice receiving irradiation limited to the primary tumour together with anti-CTLA-4 with delayed TDLN RT exhibited greater infiltration of effector CD8<sup>+</sup> T cells within both the tumour and the TDLNs, improved tumour control, and prolonged overall survival. These results were obtained with a single 15-Gy fraction and were reproduced using a 3 x 8-Gy regimen. As multiple RT fractions have been shown to induce stronger immune stimulation than a single fraction, our protocol proposes hypofractionated RT delivered exclusively to the primary tumour and delayed TDLN RT [234, 456]. The dose per fraction must be sufficient to induce adequate tumour cell destruction and trigger the release of neoantigens and damage-associated molecular patterns required for CD8<sup>+</sup> T-cell priming [235]. Therefore, conventional fractionation (1.8 – 2 Gy) was not retained. Conversely, very high doses (>12 Gy per fraction) impair RT-induced antitumour immunity through induction of the TREX1 exonuclease, which degrades cytosolic DNA and limits cGAS–STING pathway activation and interferon- $\beta$  secretion [233]. For these reasons, a moderate hypofractionated regimen of 3 x 5 Gy was selected. Higher RT doses, when used in the clinical setting, also raise concerns regarding RT-induced toxicity, particularly given that pelvic TDLNs (which encompass the rectal tumour

region) will subsequently be irradiated. For example, based on published dose constraints, a maximum of 50% of the bladder should receive <60 Gy under normofractionation, and the small-bowel volume receiving 45 Gy should remain <150 mL [457]. For delayed pelvic irradiation, a conventional long-course schedule (25 x 1.8 Gy) is proposed in order to remain consistent with regimens that have demonstrated clinical efficacy and are supported by current recommendations [94, 100, 103, 115]. The combination of a 3 x 5-Gy primary tumour boost with 25 x 1.8-Gy pelvic irradiation corresponds to an estimated biologically effective dose of approximately 92 Gy ( $\alpha/\beta = 5.06$ ), equivalent to 66 Gy in 2-Gy fractions, thus representing a dose escalation relative to current standards and potentially increasing tumour control probability [162, 277, 320]. However, simple summation of these two regimens does not account for the temporal gap between them, which may result in a lower effective biologically effective dose [458].

The first RT phase will aim to precisely target the rectal tumour while preserving the integrity of the TDLNs. In rectal cancer, the first lymphatic relay consists of the mesorectal lymph nodes, which directly surround the rectum and the primary tumour [26]. Consequently, maximal restriction of the irradiated volume is a key objective to minimise inadvertent TDLN irradiation. Among the available strategies, cone-beam CT-guided online adaptive RT, discussed in Section 3.1 of this work, allows a substantial reduction of planning target volume (PTV) margins, from 14.2 mm (antero-posterior), 5.0 mm (left-right), and 12.3 mm (supero-inferior) with non-adaptive RT to 3.3 mm, 3.2 mm, and 8.7 mm, respectively, with online-adaptive RT. In addition, daily adaptation enables safe dose escalation to the

primary tumour without increasing the risk of pelvic toxicity [276]. Magnetic resonance-guided RT represents another promising approach, allowing direct visualisation of the rectal tumour and further potential PTV margin reduction, thereby improving TDLN preservation [302]. As CBCT-guided online adaptive RT and MRI-guided RT are not widely accessible, an alternative strategy may consist of heterogeneous irradiation of the tumour volume. In preclinical models, combining low-dose (2 Gy) and high-dose (16 Gy) irradiation to distinct intratumoural subregions, in association with anti-PD-1 therapy, resulted in superior tumour control and survival compared with uniform 2-Gy or 16-Gy irradiation or partial high-dose irradiation alone [264]. Whether these findings can be translated into the clinical setting remains to be determined. The volume for subsequent pelvic irradiation will be defined according to international recommendations [161].

In the current initial version of the protocol, RT to the primary tumour is scheduled during the second cycle of immunotherapy. This sequencing allows immediate initiation of systemic treatment after patient inclusion, thereby avoiding delays related to RT planning, which typically requires one to two weeks. However, from an immunological standpoint, this sequence may compromise RT-immunotherapy synergy [265, 266]. Indeed, initiating immunotherapy before RT has been associated with increased radiosensitivity and depletion of CD8<sup>+</sup> T cells, whereas delivering RT first enhances their proportion and functional activation in both systemic circulation and the local tumour microenvironment. This has been linked to reduced local and distant tumour control when immunotherapy is administered first [265]. A recent preclinical study further suggested that the

optimal interval for introducing immunotherapy after RT is short and narrow, with maximal efficacy observed when immunotherapy is initiated approximately 5 days after irradiation [266]. Accordingly, further refinement of the proposed protocol would preferentially favour initiation with tumour-only RT followed by immunotherapy, rather than the reverse sequence. Finally, long-course pelvic RT will be delivered 2 ( $\pm$  1) weeks after completion of concomitant immunotherapy and mFOLFIRINOX, in accordance with the PRODIGE-23 regimen.

#### **3.3.4. Immunotherapy considerations**

The choice of immunotherapy agent and its timing of administration within this protocol require careful consideration in order to maximise the likelihood of achieving a complete response while avoiding an unacceptable increase in toxicity.

Anti-PD-(L)1 agents are by far the most widely used immune checkpoint inhibitors in clinical practice and are approved across multiple indications. In locally advanced MSI-H rectal cancer, anti-PD-1 therapy with dostarlimab is currently recommended as a standalone treatment and has been reported to induce clinical complete responses in up to 100% of patients [81, 94]. In contrast, outcomes in MSS disease are substantially more limited. Prospective phase II trials and meta-analyses report clinical complete response rates of less than 30% with anti-PD-1 monotherapy in MSS colorectal cancer [74, 151, 152]. Nevertheless, when anti-PD-1 therapy is combined with neoadjuvant long-course radiotherapy in MSS rectal cancer,

a reduction in intratumoural exhausted T cells and immunosuppressive M2 tumour-associated macrophages has been observed compared with baseline [74]. These findings suggest that anti-PD-1-based combinations are biologically active in MSS colorectal cancer but may be insufficient to achieve robust clinical efficacy. Potentiation of this immune response through a more immunostimulatory RT regimen may therefore represent a rational strategy to improve treatment outcomes. Importantly, the combination of anti-PD-1 therapy with hypofractionated RT (5 Gy per fraction), modified FOLFIRINOX (outside the rectal cancer setting), or long-course chemoradiotherapy has not been associated with excessive toxicity, supporting the feasibility of its integration into the proposed protocol [74, 459, 460].

Anti-CTLA-4 therapy also warrants consideration, as it was used (either alone or in combination with anti-PD-1) in preclinical studies demonstrating that delaying irradiation of tumour-draining lymph nodes relative to primary tumour RT can restore immunotherapy efficacy [249]. In the clinical setting, however, the addition of anti-CTLA-4 to anti-PD-1 during long-course chemoradiotherapy did not significantly increase complete response rates in a prospective phase II study of 50 patients with locally advanced rectal cancer, the majority of whom had MSS disease, compared with patients treated without immunotherapy (n = 30), while not increasing the incidence of adverse events [461].

Other immune checkpoint inhibitors, including anti-LAG-3, anti-TIM-3, and anti-TIGIT antibodies, are currently under investigation; however, none has

yet been validated in rectal cancer [462-464]. An alternative strategy involves the use of immunostimulatory agonists designed to enhance pro-immunogenic immune responses [463]. Among these agents, CD40 agonists are of particular interest. Mitazalimab, a CD40 agonistic antibody, promotes antigen presentation by dendritic cells, thereby enhancing CD8<sup>+</sup> T-cell priming and infiltration into the tumour immune microenvironment and ultimately mediating tumour cell death [465]. This mechanism is especially relevant in immunologically “cold” tumours, such as MSS locally advanced rectal cancer, which are characterised by low baseline immune infiltration, in contrast to dMMR/MSI-H tumours that display a “hot” immune microenvironment with abundant T-cell infiltration [82, 465]. In a phase Ib/II trial in metastatic pancreatic cancer, mitazalimab in combination with mFOLFIRINOX achieved an objective response rate of 40%, with a median response duration of 12.5 months, outperforming historical outcomes with chemotherapy alone while maintaining a safety profile comparable to mFOLFIRINOX monotherapy [466]. Although mitazalimab has not yet been evaluated in locally advanced rectal cancer, another CD40 agonist, sotigalimab, has demonstrated encouraging results in this setting. In the phase II INNATE trial, patients treated with short-course RT followed by consolidation chemotherapy (5-fluorouracil and oxaliplatin) in combination with sotigalimab achieved a higher pathological complete response rate (4/18, 22.2%) compared with patients treated without sotigalimab (1/7, 14.3%) [467]. Preclinical studies further support this approach, demonstrating improved survival with the combination of CD40 agonists and hypofractionated RT compared with either modality alone. These findings

provide a strong biological rationale for the selected boost regimen of 3 x 5 Gy in the proposed protocol [468].

The duration of immunotherapy administration and the number of treatment cycles represent critical parameters that must balance sufficient exposure to achieve optimal efficacy with the need to limit immune-related toxicity [469]. To exploit the immunostimulatory effects induced by RT, and based on preclinical evidence, initiation of immunotherapy a few days after RT appears to be optimal [266]. Conversely, because irradiation of TDLNs has been shown to abrogate immunotherapy efficacy, administration of immunotherapy during long-course chemoradiotherapy may be not advisable [247, 248, 250, 251]. Clinical experience from neoadjuvant immunotherapy trials in other tumour types further suggests that prolonged exposure to immune checkpoint inhibitors is not required to achieve meaningful clinical benefit. In resectable non-small cell lung cancer, a phase III trial demonstrated that three cycles of nivolumab, an anti-PD-1 antibody, administered concomitantly with a platinum-based doublet as neoadjuvant therapy significantly improved pathological complete response, event-free survival, and overall survival compared with chemotherapy alone [470]. Preclinical models also indicate that extending immunotherapy beyond this window may be counterproductive. Although prolonged treatment (six cycles) increased the proportion of activated (CD69<sup>+</sup>) and proliferating (Ki67<sup>+</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cells, it did not translate into improved survival and was associated with an increased risk of immune-related adverse events. These findings suggest that extended exposure may induce detrimental systemic immune perturbations[471]. Whether immunotherapy should be

administered concurrently with mFOLFIRINOX is addressed in the following section.

### **3.3.5. Chemotherapy considerations**

The proposed protocol is based on the PRODIGE-23 regimen, which incorporates mFOLFIRINOX as neoadjuvant chemotherapy, followed by long-course radiotherapy with concomitant capecitabine [115]. The use of oxaliplatin-based doublet chemotherapy in the neoadjuvant setting has also been investigated in other landmark total neoadjuvant therapy trials [102, 103, 472]. The ongoing phase III JANUS trial directly compares doublet versus triplet chemotherapy regimens within a total neoadjuvant treatment strategy for locally advanced rectal cancer, with clinical complete response and disease-free survival as key endpoints [473]. The addition of fluoropyrimidine to long-course RT reduce local relapse rate [105, 106]. However, oxaliplatin-based doublet does not improve local control compared to a fluoropyrimidine alone and increase grade  $\geq 3$  adverse events suggesting that the preferred concomitant treatment should be a single fluoropyrimidine [107-109]. Fluoropyrimidine are radiosensitiser through the killing of cells that have inappropriate progression through S phase of the cell cycle [474].

As discussed in the previous section, it remains unclear whether immunotherapy should be combined with mFOLFIRINOX. Among the agents included in the FOLFIRINOX regimen, oxaliplatin has the strongest evidence for inducing immunogenic cell death. In preclinical models, oxaliplatin has



been shown to modulate antitumour immune responses through several mechanisms. Notably, oxaliplatin induces the expression of ADAR1, an adenosine deaminase involved in RNA editing, thereby promoting neoantigen generation [475]. In addition, oxaliplatin triggers the release of damage-associated molecular patterns, particularly adenosine triphosphate, which promotes monocyte recruitment and induces interleukin-1 $\beta$  secretion by dendritic cells, an essential step for interferon- $\gamma$ -dependent CD8<sup>+</sup> T-cell activation [476]. Interestingly, preclinical data indicate that anti-PD-L1 immunotherapy followed by oxaliplatin results in increased CD8<sup>+</sup> T-cell infiltration and reduced regulatory T cells within the tumour, tumour-draining lymph nodes, and spleen, compared with either monotherapy or alternative sequencing strategies (concomitant administration or oxaliplatin followed by anti-PD-L1). In this model, survival and tumour control were significantly improved with the sequence of anti-PD-L1 therapy followed by oxaliplatin [477]. 5-fluorouracil has also been shown to enhance releasing of calreticulin, another damage-associated molecular pattern, thereby promoting T-cell infiltration into the tumour microenvironment [478].

The chemotherapy dose within chemo-immunotherapy combinations is another key parameter. Chemotherapy can cause dose-dependent myelosuppression, and when delivered at the maximum tolerated dose, treatment-related cytopenias may decrease the efficacy of immunotherapy. Conversely, dose reduction can mitigate myelosuppression and may restore synergistic interactions with immunotherapy [479].

### **3.3.6. Conclusion**

In MSS locally advanced rectal cancer, increasing the clinical complete response rate is a key objective to expand eligibility for organ-preservation strategies. A clinical study protocol is proposed and is designed to enhance complete response rates by integrating immunotherapy, neoadjuvant chemotherapy, and a hypofractionated RT boost to the primary tumour, while delaying irradiation of tumour-draining lymph nodes. However, combining these modalities is inherently complex, as it requires careful consideration of multiple parameters and is mostly supported by preclinical evidence, given that this approach has not yet been extensively evaluated in humans. Based on the available literature, a refinement of the initial experimental protocol is proposed (

Figure 3.3.3).

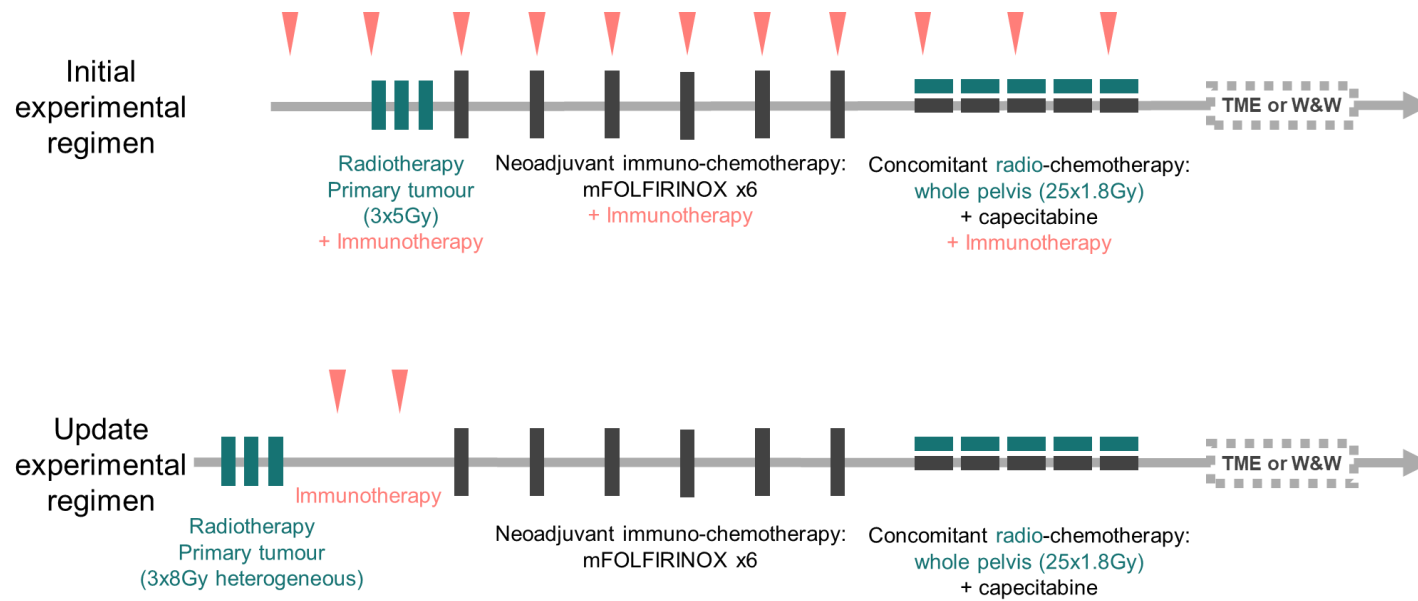


*Figure 3.3.3: Updated study protocol. Compared to the initial proposed regimen, the modified approach would use immunotherapy after radiotherapy to the primary tumour to enhance the synergy between these two modalities that is expected to be higher than concomitant or immunotherapy first administration. Similarly, immunotherapy before neoantigen generating chemotherapy is the optimal sequence regarding these two modalities. This will lead to a limited administration of immunotherapy, which has been demonstrated to be effective in neoadjuvant setting and which limits the risk of immune-related adverse events. In order to spare tumour draining lymph node during the primary tumour radiotherapy, a heterogeneous irradiation can be considered. It can also allow the escalation of the radiotherapy dose to a radiotherapy regimen optimised to induce the strongest immune reaction as possible. TME: Total mesorectal excision, W&W: Watch and Wait.*



## Chapter 4

# Metastatic colorectal cancer

#### **4.1. Markerless liver online adaptive stereotactic radiotherapy: Feasibility analysis**

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## Highlights

- Markerless liver SABR is feasible using CBCT-guided online-adaptive RT
- Image-guided DIR with rigid mapping is the best target volume propagation strategy
- PTV should include the automatic propagation error, leading to a larger volume
- Daily adaptation ensures OAR safety and improves target coverage despite larger PTV



## **Abstract**

### *Objective*

Radio-opaque markers are recommended for image-guided radiotherapy in liver stereotactic ablative radiotherapy (SABR), but their implantation is invasive. We evaluate in this *in-silico* study the feasibility of cone-beam computed tomography (CBCT)-guided stereotactic online-adaptive radiotherapy (oART) to propagate the target volumes without implanting radio-opaque markers and assess its consequence on the margin that should be used in that context.

### *Approach*

An emulator of a CBCT-guided stereotactic oART-dedicated treatment planning system was used to generate plans for 32 liver SABR patients. Three target volume propagation strategies were compared, analysing the volume difference between the  $GTV_{\text{Propagated}}$  and the  $GTV_{\text{Conventional}}$ , the vector lengths between their centres of mass ( $l_{\text{CoM}}$ ), and the 95<sup>th</sup> percentile of the Hausdorff distance between these two volumes (HD95). These propagation strategies were: (1) structure-guided deformable registration with deformable GTV propagation; (2) rigid registration with rigid GTV propagation; and (3) image-guided deformable registration with rigid GTV propagation. Adaptive margin calculation integrated propagation errors, while interfraction position errors were removed. Scheduled plans ( $\text{Plan}_{\text{Non-adaptive}}$ ) and daily-adapted plans ( $\text{Plan}_{\text{Adaptive}}$ ) were compared for each treatment fraction.

### *Main results*

The image-guided deformable registration with rigid GTV propagation was the best propagation strategy regarding to  $l_{CoM}$  (mean: 4.3 +/- 2.1 mm), HD95 (mean 4.8 +/- 3.2 mm) and volume preservation between  $GTV_{Propagated}$  and  $GTV_{Conventional}$ . This resulted in a planning target volume (PTV) margin increase (+69.1% in volume on average). Online adaptation ( $Plan_{Adaptive}$ ) reduced the violation rate of the most important dose constraints ("priority 1 constraints", 4.2 versus 0.9 %, respectively;  $p < 0.001$ ) and even improved target volume coverage compared to non-adaptive plans ( $Plan_{Non-adaptive}$ ).

### *Significance*

Markerless CBCT-guided stereotactic oART for liver tumours is feasible using Image-guided deformable registration with rigid GTV propagation. Despite the cost in terms of PTV volumes, daily adaptation reduces constraints violation and restores target volumes coverage.

#### **4.1.1. Introduction**

Surgery is the cornerstone of the treatment of localised hepatocarcinoma or liver metastases from oligometastatic colorectal cancers since it has a major impact on survival [39, 480]. However, in some cases, surgery cannot be proposed due to patients' comorbidities or because it comes at a large liver cost for the patient. Stereotactic ablative body radiotherapy (SABR) can be a good alternative with a high control rate and low adverse events [39, 138, 480, 481]. However, liver SABR still faces some technical issues, especially in relation to tumour visualisation and motion management due to breathing or physiologic abdominal organ motion. Liver tumours often present with similar density to the surrounding tissue and are therefore hardly identified during the usual cone-beam computed tomography (CBCT) performed for image-guided radiotherapy. Image-guided RT is now routine and enables accurate patient setup with respect to tumour position and prevents geographic misses of the tumour that would jeopardise the treatment efficacy or lead to undesirable adverse events [180, 482]. Therefore, radio-opaque markers are frequently implanted percutaneously around the liver tumour and used as surrogates of the tumour position on the CBCT [181, 483-485]. As with other percutaneous procedures, this can lead to complications such as abdominal pain or bleeding, justifying the patient's surveillance, or even tumour cells spreading [182, 486, 487]. Moreover, when radio-opaque marker implantation is unfeasible due to an inaccessible anatomical location, lack of tumour visibility on ultrasonography, or patients' frailties, liver SABR might be compromised. Strategies that eliminate the need for invasive procedures (radio-opaque markers or

lipiodol intra-tumoral injection), such as the use of an internal liver volume defined on a four-dimensional CBCT to guide the image-guided RT, are therefore investigated to reduce the invasiveness of the liver SABR [488]. Magnetic resonance image (MRI)-guided radiotherapy may be an adequate solution since it can localise the tumour without the need for markers, enables online-adaptive radiotherapy to compensate for interfraction tumour motion, and allows management of intrafraction motion using real-time MRI guidance. However, MRI-guided radiotherapy solutions are expensive, rarely available, and require dedicated education and installations for their use [183-185]. On the other hand, CBCT-guided stereotactic online-adaptive radiotherapy (oART) has been harnessed and recently implemented thanks to a new ring gantry linear accelerator equipped with a fast and high-quality volumetric CBCT imaging system and an on-board treatment planning system (ETHOS; Varian Medical Systems, Palo Alto, Calif., USA) [190, 192, 313, 314]. In adaptive workflows based on the CBCT acquired at each treatment sessions, artificial intelligence algorithms allow fast automatic delineation of organs at risk (OARs), called influencers, automatic influencers-guided propagation of target volumes, and automatic daily plan re-computation [190, 192]. Online-adaptive radiotherapy might offer new possibilities with its automatic volume propagation algorithms. This might compensate for the lack of tumour visibility on CBCT, making consequently radio-opaque markers futile. Also, daily planning can potentially result in better OARs sparing and target volume coverage for liver SABR.

*We performed here an in-silico analysis to evaluate CBCT-guided stereotactic oART performance in markerless liver SABR, without access to MRI daily images. This analysis follows a multistep approach (*

Figure 4.1.1). We investigated first the best strategy for target volume propagation (step 1). Then we evaluated if the presence of radio-opaque markers impacted the accuracy of target volume propagation (step 2). From that point, we had to integrate the consequent target volume propagation residual errors within a planning target volume (PTV) margin calculation based on van Herk's recipe (step 3) [174, 489]. We applied this new PTV margin to the tumours and assessed in the CBCT-guided stereotactic oART environment the adaptive plans, which were compared to conventional non-adapted plans for target volume coverage and OARs protection (step 4).

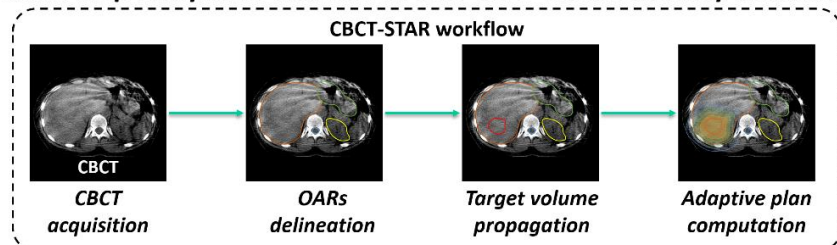
## **4.1.2. Material and Methods**

### **4.1.2.1. Patient selection**

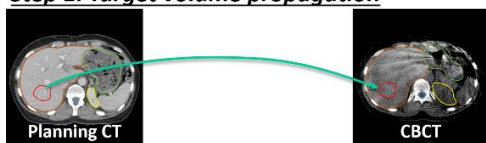
We retrospectively reviewed patients treated by SABR for liver tumours in two institutions between July 2010 and June 2021. Inclusion criteria were patients over the age of 18 years, a unique irradiated tumour, and access to both planning and CBCT images. Twenty-one patients with radio-opaque markers were included in the first cohort (cohort with markers). We included only patients with markers located at most 1 cm outside the PTV in order to limit the uncertainties in predicting tumour position based on marker localisation. Eleven patients without radio-opaque markers were included in the second cohort (cohort without markers). In this cohort without markers,

the tumour was directly visible on CBCT because it was located at the periphery of the liver and distorted the liver capsule. All the planning CTs and CBCTs were collected for each patient. Table 4.1.1 reports the patients and the treatment characteristics of each cohort. This retrospective study was conducted in accordance with the declarations of Helsinki and was approved by the ethics committees of Cliniques Universitaires Saint-Luc and CHU UCL Namur, site Sainte Elisabeth.

## Multi-step analysis for markerless liver CBCT-STAR feasibility evaluation

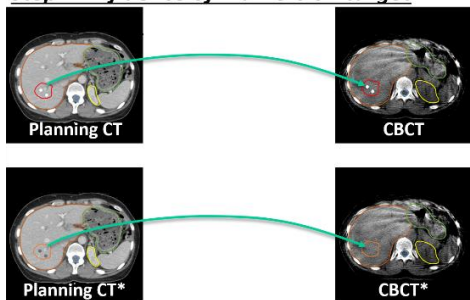


### Step 1. Target volume propagation



- Comparison between 3 propagation strategies:
- Structure-guided DIR/Deformable propagation
  - Rigid registration/Rigid propagation
  - **Image-guided DIR/Rigid propagation**  
= best strategy

### Step 2. Influence of markers on target



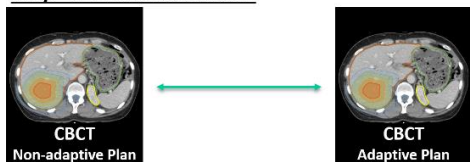
- Comparison between:
- The volume propagated from the planning CT to the CBCT
  - The volume propagated from the planning CT\* to the CBCT\* (Voxels within markers' volume are overwritten to liver density)

### Step 3. PTV margin computation



Integration of propagation errors in the PTV margin

### Step 4. Plan evaluation



Comparison between Non-adaptive and adaptive plans

*Figure 4.1.1: Summary of the CBCT-guided stereotactic oART workflow with the different steps of the analysis and the use of each image data set. Step 1 aimed to evaluate the accuracy of target volume propagation by the ETHOS®. For that purpose, three different propagation strategies were compared using data from 21 liver SABR patients with markers (174 CBCTs and 21 planning CTs). Step 2 evaluated the impact of the presence of high-density structures (i.e., radiopaque markers) on the target volume propagation accuracy. We compared the results obtained with the best propagation strategy in the first step with those obtained on the same set of CBCTs and planning CT on which voxels within markers' volume had been overwritten to surrounding liver tissue Hounsfield units. Step 3 quantified and integrated the target volume propagation error of the ETHOS® in a PTV margin based on van Herk's recipe [174]. We used the same dataset from 21 liver SABR patients with markers and, also, data from 11 liver SABR patients without markers (75 CBCTs). Step 4 compared adaptive plans with non-adaptive plans based on target volume coverage and OARs sparing. Five adaptive and five non-adaptive sessions (prescription dose: 5 x 10 Gy) were virtually delivered and compared in 20 patients. CBCT: Cone-beam computed tomography, DIR: Deformable image registration, OARs: Organs at risks, PTV: Planning target volume, SABR: Stereotactic ablative radiotherapy, STAR: Stereotactic adaptive radiotherapy.*



|                            | Patients with<br>markers | Patients without<br>markers |
|----------------------------|--------------------------|-----------------------------|
| N                          | 21                       | 11                          |
| Mean Age (years)           | 63.4 +/- 14.1            | 68.1 +/- 10.0               |
| Gender                     |                          |                             |
| Male                       | 14                       | 5                           |
| Female                     | 7                        | 6                           |
| Primary disease            |                          |                             |
| Colorectal cancer          | 14                       | 2                           |
| Hepatocarcinoma            | 4                        | 5                           |
| Ovary cancer               | 1                        | 1                           |
| Non-small cell lung cancer | 1                        | 1                           |
| Breast cancer              | 1                        | 0                           |
| Renal cell carcinoma       | 0                        | 1                           |
| Schwannosarcoma            | 0                        | 1                           |
| Tumour location*           |                          |                             |
| Segment I                  | 5                        | 1                           |
| Segment II                 | 3                        | 1                           |
| Segment III                | 1                        | 0                           |
| Segment IV                 | 5                        | 2                           |
| Segment V                  | 1                        | 1                           |
| Segment VI                 | 1                        | 3                           |
| Segment VII                | 6                        | 2                           |
| Segment VIII               | 8                        | 2                           |

| Initial radiotherapy dose          |               |               |
|------------------------------------|---------------|---------------|
| 3 x 15 Gy                          | 9             | 3             |
| 3 x 17.25 Gy                       | 0             | 1             |
| 4 x 10 Gy                          | 0             | 2             |
| 5 x 6 Gy                           | 2             | 1             |
| 5 x 7 Gy                           | 1             | 2             |
| 5 x 10 Gy                          | 2             | 0             |
| 6 x 8 Gy                           | 6             | 1             |
| 10 x 4 Gy                          | 1             | 0             |
| 11 x 3 Gy                          | 0             | 1             |
| Mean GTV volume (cm <sup>3</sup> ) | 28.9 +/- 20.6 | 34.6 +/- 33.7 |
| N of available CBCTs               | 174           | 75            |
| N of available 4D-planning CTs     | 16            | 4             |

*Table 4.1.1: Demographic and initial treatment characteristics of the patients with and without markers. The tumour location was assessed on MRI and classified according to the Brisbane 2000 nomenclature [490]. \* Eight tumours in patients with markers and one tumour in patients without markers involved multiple hepatic segments.*

#### **4.1.2.2. Initial treatment and image characteristics**

In the first institution, a 3- to 10-phases four-dimension (4D)-planning CT (General Electric RT16, General Electric Company, Boston) with a slice thickness of 2.5 mm was acquired in the supine position for liver SABR planning. Thoracic contention was assured by a thermoplastic mask without

abdominal compression. The gross tumour volume (GTV) was delineated using multimodality imaging (contrast-enhanced CT, liver MRI, and <sup>18</sup>fluorodeoxy-glucose positron emission tomography if available). The tumour was delineated on all 4D-planning CT phases, and the contours were merged to create an internal target volume. PTV was created by adding an isotropic margin of 5 mm around the internal target volume. Treatments were planned on the Eclipse planning system (Varian Medical Systems, Palo Alto, CA, USA) and delivered on two linear accelerators (Truebeam®, Varian Medical Systems, Palo Alto, CA, USA, and Novalis TX, Varian Medical Systems, Palo Alto, CA, USA). For the image-guided RT process, a 3D-CBCT was performed before each session and, in some cases, a second 3D-CBCT was made between two arcs to compensate for the intra-fraction variability.

In the second institution, a 10-phases contrast-enhanced 4D-planning CT (Aquilion LB, Canon medical systems corporation, Japan) was acquired in the same position but with audio-coaching to regularise the breathing pattern. The slice thickness was 2 mm. A vacuum bag guaranteed contention, and abdominal compression was applied to limit respiratory motion. A Mid-Position CT was reconstructed by non-rigidly deforming the 10 phases of the 4D-planning CT to generate images that represents the time-weighted average anatomy of the patient over the entire breathing cycle. The GTV was again delineated using multimodality imaging. The Mid-Position PTV margin was computed according to van Herk's recipe [174]. Treatments were planned on the Raystation Planning system (clinical version 9B, RaySearch Laboratories, Stockholm, Sweden). Radiotherapy sessions were delivered on one linear accelerator (Halcyon®, Varian Medical Systems, Palo Alto, CA,

USA) with an audio-coaching ensuring the reproducibility of the planning conditions. One daily CBCT was performed at the start of the session for the image-guided RT process. In both institutions, delineated OARs included the oesophagus, stomach, duodenum, small and large bowels, heart, spinal canal, liver, great vessels, kidneys right and left, chestwall, and skin.

All the CBCTs retrieved from these two institutions were included in the target volume propagation analysis.

#### *4.1.2.3. CBCT-guided stereotactic oART in-silico workflow*

Routinely, for all CBCT-guided adaptive treatments performed with ETHOS, the delineation of target volumes and OARs is performed on the planning CT, and a first plan is prepared, considering the dose prescription but also pre-defined clinical goals for OARs. Afterwards, during the adaptive sessions, the OARs close to the tumour and assigned as influencers are automatically delineated with artificial intelligence-based automatic segmentation and possibly adjusted by a radiation oncologist. Those influencers lead to the deformation field between the planning CT and the CBCT, generating consequently the target volumes using a deformable image registration (DIR) on the CBCT. In addition, a synthetic CT is generated using an image-guided DIR between the planning CT and the CBCT (Velocity™, Varian Medical Systems, Palo Alto, CA, USA) to account for the electron density changes and to create the remaining non-influencer OAR structures matching the anatomy of the day. Finally, a daily-adapted plan (Plan<sub>Adaptive</sub>) is computed based on this synthetic CT and the contours of the day. A

comparison is made between the Plan<sub>Adaptive</sub> and the Plan<sub>Non-adaptive</sub>, which is the initial planning CT plan recalculated on the daily synthetic CT and contours. The best plan is chosen by the radiation oncologist based on dosimetric parameters before being delivered. For the purpose of this study, we followed a similar procedure to mimic clinical adaptation thanks to the virtual environment of the emulator provided by ETHOS vendors, which contains the CBCT-guided stereotactic oART treatment planning system as well as the online-adaptive radiotherapy software.

During the virtual adaptive sessions in the emulator environment, the CBCTs were retrieved from the patient database as substitutes for the daily imaging. The stomach, the kidney right, the lung right, the lung left, and the spinal canal were chosen as influencers due to their close anatomical relationship with the liver.

All the virtual CBCT-guided stereotactic oART sessions data were retrospectively downloaded through the emulator.

#### *4.1.2.4. Target volume propagation evaluation (step 1)*

This first analysis aimed to determine the best strategy for the propagation of the liver tumour by measuring the position difference between the GTV propagated by the online CBCT-guided stereotactic oART algorithms (GTV<sub>Propagated</sub>) and the expected position of the tumour on the daily CBCT.

The definition of this “expected GTV position” was complex due to the lack of visibility of the tumour on the CBCT. In the absence of daily MRI, we had

no GTV groundtruth to rely on. Therefore, we decided to base the GTV conventional definition on what is currently accepted during common image-guided RT workflows and non-adaptive treatments, which is the GTV position we would have accepted after a classical rigid image-guided RT protocol performed during a conventional liver SABR procedure (GTV<sub>Conventional</sub>).

During this conventional liver SABR workflow, a rigid registration is performed between the planning CT and the CBCT of the day by a radiation oncologist. This rigid registration is guided by the alignment of the radio-opaque markers visualised on both the planning CT and the CBCT, or by anatomical alignment in the absence of markers whenever the tumour is visible or a liver capsule distortion is observed. Afterwards, a table displacement based on the rigid registration values is performed in order to rigidly propagate the planning GTV on the CBCT. This determined the GTV<sub>Conventional</sub> position of the day. Such a GTV<sub>Conventional</sub> has been retrieved for all the CBCTs included in our study following this method.

In our analysis, we evaluated the ETHOS capacity to correctly propagate the target volumes thanks to its automated algorithms. With the current clinical version of the ETHOS, two strategies to obtain the GTV<sub>Propagated</sub> were available for our analyses. One strategy refers to the conventional non-adaptive workflow, with an automatic rigid registration between the planning CT and CBCT and a rigid propagation of the GTV (Rigid registration/Rigid propagation). The second strategy relies on a *structure-guided* DIR between the planning CT and CBCT using influencers, then

applying the resulting vector field to *deform* and propagate the GTV (Structure-guided DIR/Deformable propagation). This strategy is mainly dedicated to adaptive radiotherapy for pelvic tumours. The accuracy of the structure-guided DIR, which uses a non-demons algorithm [491], has been evaluated by the vendor. They automatically delineated (and corrected when needed) the volume of influencer structures and target volumes on a CBCT and a CT. The DIR was calculated between the CBCT and the CT. The CT structures were propagated to the CBCT using the DIR vector field. Mean distances between propagated and verified structures were 1.0 – 1.5 mm on average.

An additional pre-clinical propagation strategy has been provided by the vendor. This new version allowed us to first choose between *structure-guided* or *image-guided* DIR and then between *rigid*, *slice-wise rigid*, or *deformable* target volume propagation, resulting in six different combinations. A preliminary analysis enabled us to select the best propagation strategy among those six combinations, which is the *image-guided* DIR used in combination with the *rigid* GTV propagation (Image-guided DIR/Rigid propagation). In this strategy, the centre of mass of the GTV is propagated using the deformation field obtained after the image-guided DIR, and then the GTV is rigidly propagated based on its new centre of mass position. The vendor evaluated the accuracy of the image-guided DIR by comparing structures delineated on a CBCT and structures delineated on the CT, which were propagated on the CBCT using the image-guided DIR. These structures included well-visible structures on both images, which a deformable registration algorithm should be able to match accurately (i.e.,

body outline, lungs, spinal canal, and ribs for the upper abdominal region). This method was run in around 50 cases and showed a mean contour distance of 1.0 – 1.5 mm on average for the selected structures. In addition, the consistency of the vector field was checked for areas between verified structures.

As a result, we tested within our cohort the two strategies that were available in the current clinical version (Structure-guided DIR/Deformable propagation and Rigid registration/Rigid propagation) and the best strategy of the pre-clinical version of the ETHOS (Image-guided DIR/Rigid propagation, see Appendix 8.1).

*Virtual CBCT-guided stereotactic oART sessions were delivered for the 21 patients in the cohort with markers using the 3 propagation strategies on the 174 available CBCTs (*

Figure 4.1.1, step 1). Structure sets were collected after virtual CBCT-guided stereotactic oART sessions, and GTV<sub>Propagated</sub> was compared to GTV<sub>Conventional</sub> by using three metrics: (1) the length of the vector between the centres of mass of the GTV<sub>Propagated</sub> and the GTV<sub>Conventional</sub> in millimetres ( $I_{CoM}$ , surrogate of the translation difference); (2) the 95<sup>th</sup> percentile of the Hausdorff distance between these two structures, which corresponds to the greatest distance between these volumes among their 95% closest voxels. (HD95, surrogate of the rotation difference); (3) the volume difference between the two segmentations in percent. The propagation strategy was considered to be the finest when the  $I_{CoM}$ , the HD95, and the volume difference results tended towards zero, assuming that the shape and volume of the tumours remained constant during the whole course of the SABR treatment.



#### 4.1.2.5. *Impact of radio-opaque markers assessment (step 2)*

Since the image-guided DIR strategies for propagation rely on Hounsfield units values of the image sets, we performed an additional analysis in order to evaluate the potential impact of radio-opaque markers on the DIR accuracy. We aimed to exclude a possible excessive influence of these high-density structures on the mapping of the voxels' Hounsfield units between the planning CT and the CBCT that is performed in the image-guided DIR strategy.

*We generated planning CTs and CBCTs of the cohort with markers where voxels within markers' volume were overwritten to 100 - 150 Hounsfield units, which was similar to values of the surrounding liver tissue. Then, we computed the propagation error ( $I_{COM}$ ) by using these overwritten images and compared it with propagation error values obtained when using native images with markers present (*

Figure 4.1.1, step 2). If these values were not significantly different, we could conclude that the influence of the radio-opaque markers was not significant in the result of the propagation strategy.

#### 4.1.2.6. *PTV margin computation (step 3)*

We aimed to compute a modified Mid-Position PTV margin that would include the CBCT-guided stereotactic oART propagation errors from the selected target volume propagation strategy, according to the van Herk's recipe [174].

The original van Herk's formula intends to integrate systematic and random errors into a PTV margin, considering a moving target volume. The formula is:

$$M_{PTV_{Mid-Position}} = \alpha \sqrt{\Sigma_{TM}^2 + \Sigma_{BL}^2 + \Sigma_{Setup}^2 + \Sigma_D^2} + \beta \sqrt{\sigma_{TM}^2 + \sigma_{BL}^2 + \sigma_{Setup}^2 + \sigma_p^2} - \beta \sigma_p \quad (Eq. 4.1.1)$$

The coefficient  $\alpha$  and  $\beta$  are set at 2.5 and 1.64 to ensure that the GTV coverage is at least 95% of the prescribed dose for 90% of patients. The standard deviations of systematic and random errors are represented by  $\Sigma$  and  $\sigma$  respectively. "TM", "BL", "Setup", "D" and "p" indexes refer to tumour motion, baseline shift, patient setup variability, delineation uncertainty, and penumbra, respectively (Eq. 4.1.1). Values for baseline shift and delineation errors were taken from the literature and adapted regarding accelerator/treatment planning system specificities and institutional protocols [492-494]. The penumbra value was determined according to the method described by Wanet et al. and also adapted to the accelerator/treatment planning system specificities [173]. The computation of systematic and random errors for tumour motion remained identical to the method used in our clinical practice, based on Mid-position CT generation and 4D-planning CT. Integrating the tumour motion and baseline shift errors in the Mid-Position PTV margin allowed for compensation of the intrafraction motion of the tumour, which is mainly related to the

respiratory motion of the patient. The detailed values of these errors are reported in Appendix 8.2.

*In this particular CBCT-guided stereotactic oART workflow, daily adaptation allowed us to overcome errors due to patient setup variations, which were set to zero. However, we still needed to integrate both the systematic ( $\Sigma_{PE}$ ) and the random ( $\sigma_{PE}$ ) components of the target volume propagation errors in the new modified PTV (mPTV) margin, which depend on the selected propagation strategy (Eq.4.1.2,*

Figure 4.1.1, step 3). We computed these propagation errors by quantifying the distance in millimetres between the centres of mass of the GTV<sub>Propagated</sub> and the GTV<sub>Conventional</sub> in the three dimensions and reported the systematic and random propagation errors according to the method described by van Herk et al [489]. Accordingly, the systematic error ( $\Sigma_{PE}$ ) was the standard deviation of the distribution of the mean distances per patient. The random error ( $\sigma_{PE}$ ) was the root mean square of the distribution of the distance standard deviations per patient. These propagation errors were computed by using data from both cohorts with and without markers.

The final margin formula was thus adapted as follows:

$$M_{mPTV_{MidP}} = \alpha \sqrt{\Sigma_{TM}^2 + \Sigma_{BL}^2 + \Sigma_D^2 + \Sigma_{PE}^2} + \beta \sqrt{\sigma_{TM}^2 + \sigma_{BL}^2 + \sigma_p^2 + \sigma_{PE}^2} - \beta \sigma_p \text{ (Eq. 4.1.2)}$$

#### 4.1.2.7. Plan evaluation (step 4)

Virtual plans were generated for the 20 patients for whom a 10-phases 4D-planning CT was available (16/21 patients in the cohort with markers, 4/11

in the cohort without markers). All plans were generated by a single operator. A dose of 50 Gy in 5 fractions was prescribed. An optimised PTV was used for planning purposes and was created by excluding OARs plus 2 mm from the PTV. Plans were optimised to ensure that 100% of the GTV and the optimised PTV received more than 98% and 95% of the prescribed dose, respectively. Also, a GTV dose escalation up to 120% of the prescribed dose was recommended, with the steepest dose gradient possible. This gradient was obtained by restricting the dose received by 2% of the optimised PTV minus GTV to 110% of the prescribed dose. OAR dose constraints (described in Table 4.1.2) were grouped in Priority 1, Priority 2, and Priority 3 levels according to whether they were more, equally, or less important than target volume coverage, respectively. A reduction of the dose prescribed to the target volumes was allowed to meet OAR constraints.

| Organs at risk | Dosimetric parameters        | Goals                | Priority |
|----------------|------------------------------|----------------------|----------|
| Chestwall      | D0.1 cm <sup>3</sup>         | < 4300 cGy           | 3        |
|                | D30 cm <sup>3</sup>          | < 3000 cGy           | 3        |
|                | V3500 cGy                    | < 1 cm <sup>3</sup>  | 3        |
| Duodenum       | D0.1 cm <sup>3</sup>         | < 3200 cGy           | 1        |
|                | V1250 cGy                    | < 10 cm <sup>3</sup> | 1        |
|                | V1800 cGy                    | < 5 cm <sup>3</sup>  | 1        |
| Esophagus      | D0.1 cm <sup>3</sup>         | < 3200 cGy           | 1        |
|                | V1950 cGy                    | < 5 cm <sup>3</sup>  | 1        |
| Great Vessels  | D0.1 cm <sup>3</sup>         | < 5300 cGy           | 1        |
|                | V4700 cGy                    | < 10 cm <sup>3</sup> | 2        |
| Heart          | D0.1 cm <sup>3</sup>         | < 3800 cGy           | 1        |
|                | V3200 cGy                    | < 10 cm <sup>3</sup> | 1        |
| Kidney Left    | At least 200 cm <sup>3</sup> | < 1750 cGy           | 3        |
| Kidney Right   |                              |                      | 3        |
| Large Bowel    | D0.1 cm <sup>3</sup>         | < 3800 cGy           | 1        |
|                | V2500 cGy                    | < 20 cm <sup>3</sup> | 1        |
| Liver - PTV    | At least 700 cm <sup>3</sup> | < 2100 cGy           | 1        |
|                | Dmean                        | < 1250 cGy           | 1        |
| Skin           | D0.1 cm <sup>3</sup>         | < 3950 cGy           | 3        |
|                | V3650 cGy                    | < 10 cm <sup>3</sup> | 3        |
| Small Bowel    | D0.1 cm <sup>3</sup>         | < 3000 cGy           | 1        |
|                | D5 cm <sup>3</sup>           | < 1600 cGy           | 1        |
| Spinal Canal   | D0.1 cm <sup>3</sup>         | < 2300 cGy           | 1        |
| Stomach        | D0.1 cm <sup>3</sup>         | < 3200 cGy           | 1        |
|                | D10 cm <sup>3</sup>          | < 1800 cGy           | 1        |

*Table 4.1.2: Organs at risk dose constraints used for liver stereotactic (adaptive) radiotherapy. PTV: Planning target volume.*

*A first plan was made on the emulator with a conventional Mid-Position PTV margin (Eq.4.1.1). This plan was loaded in the emulator, and CBCT-guided stereotactic oART sessions were performed. For each session, the non-adaptive plan was systematically selected to mimic conventional non-adapted liver SABR treatment (Plan<sub>Non-adaptive</sub>). A second plan was made on the emulator, including the propagation errors into the mPTV margin to emulate a markerless workflow (Eq.4.1.2). CBCT-guided stereotactic oART sessions were delivered according to the best choice between the scheduled and adapted plans (Plan<sub>Adaptive</sub>). For all sessions (adaptive and non-adaptive), GTV and mPTV were propagated using the automatic Rigid registration/Rigid propagation strategy provided by the commercial version of the ETHOS in order to avoid large volumetric variations. For this plan analysis, the Image-guided DIR/Rigid propagation strategy was not available in the provided version of the emulator. Plan<sub>Adaptive</sub> and Plan<sub>Non-adaptive</sub> of each session were compared according to their compliance with dosimetric goals. The same optimisation parameters on target volumes and OARs were used for both plans (*

Figure 4.1.1, step 4).

#### **4.1.2.8. Time analysis**

For each CBCT-guided stereotactic oART session performed on the emulator, we reported the time elapsed between the importation of the CBCT from the database and the approval of the plan.

#### **4.1.2.9. Data analysis and Statistical considerations**

CT images, structure sets, and radiotherapy plans were extracted as DICOM files and imported into 3D Slicer version 4.11.20210226 (free open source license) for segmentation and dosimetric data collection [495]. Comparisons

of continuous variables were made using two-tailed (paired) t-tests or Wilcoxon tests for normal or non-Gaussian distributions, respectively. Dosimetric data were classified as “respected” or “violated” if the CBCT-guided stereotactic oART plans met or not the OAR doses constraints and were compared using the Fisher exact test. P-values lower than 0.05 were considered statistically significant. Statistical analyses and graphs creation were performed in R version 4.2.0.

### **4.1.3. Results**

#### ***4.1.3.1. Accuracy of target volume propagation (step 1)***

In the cohort with markers, the three propagation strategies (Structure-guided DIR/Deformable propagation, Rigid registration/Rigid propagation, and Image-guided DIR/Rigid propagation) were applied to all the available CBCTs ( $n = 174$ ). The length of the vector between the centres of mass ( $l_{CoM}$ ), the 95<sup>th</sup> percentile of the Hausdorff distance (HD95), and the volume difference between the  $GTV_{Conventional}$  and the  $GTV_{Propagated}$  by each of the propagation strategies are reported in Figure 4.1.2.

The mean ( $\pm$  one standard deviation)  $l_{CoM}$  for Structure-guided DIR/Deformable propagation, Rigid registration/Rigid propagation, and Image-guided DIR/Rigid propagation strategies was 5.3 mm ( $\pm$  2.7), 5.2 mm ( $\pm$  3.4), and 4.3 mm ( $\pm$  2.1), respectively (Figure 4.1.2). A significant difference ( $p < 0.01$ ) was obtained for the Image-guided DIR/Rigid propagation strategy compared to either the Structure-guided

DIR/Deformable propagation and the Rigid registration/Rigid propagation strategies. For the HD95, the mean ( $\pm$  one standard deviation) was 7.6 mm ( $\pm$  3.7), 4.8 mm ( $\pm$  3.0), and 4.8 mm ( $\pm$  3.2) for Structure-guided DIR/Deformable propagation, Rigid registration/Rigid propagation, and Image-guided DIR/Rigid propagation strategies, respectively (Figure 4.1.2). The HD95 values were significantly higher ( $p < 0.0001$ ) when using Structure-guided DIR/Deformable propagation compared to either the Rigid registration/Rigid propagation and the Image-guided DIR/Rigid propagation strategies (which provided similar results,  $p = 0.42$ ). These results suggested a lower propagation error when using the Image-guided DIR/Rigid propagation strategy for target volume propagation.

Regarding the volume variation, the mean volume difference compared to the  $GTV_{Conventional}$  was -5.2% ( $\pm$  20.3) when using the Structure-guided DIR/Deformable propagation strategy, which represented an important variation of the  $GTV_{Propagated}$ . The conservation of volume was however ensured by the algorithm of the two last strategies (Figure 4.1.2). Based on these three metrics values, Image-guided DIR/Rigid propagation has been considered the best propagation strategy and has therefore been used for the computation of the CBCT-guided stereotactic oART propagation errors for the mPTV margin determination (Eq. 4.1.2).



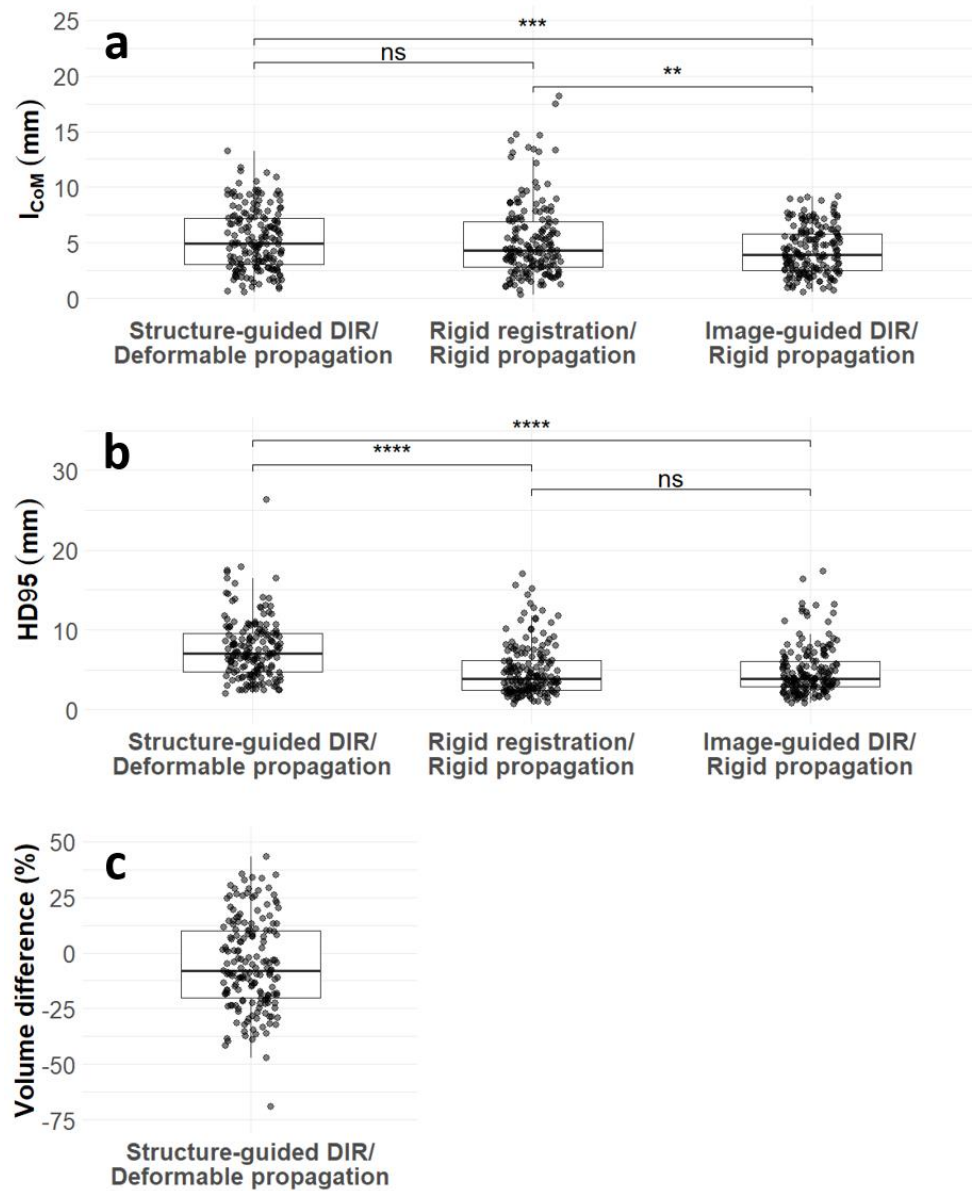


Figure 4.1.2: Accuracy of target volume propagation. The GTV was propagated on 174 CBCTs from patients with markers based on three different strategies: structure-guided DIR between planning CT and CBCT with deformable propagation of the GTV, rigid registration with rigid GTV propagation, and image-guided DIR with rigid propagation. The vector

lengths measured between centres of mass (lCoM) of GTVPropagated and GTVConventional (a) were significantly lower for the image-guided DIR with rigid propagation compared to others strategies ( $p < 0.01$ ). Also, the 95th percentile of the Hausdorff distance (HD95) values were significantly higher for the structure-guided DIR with deformable propagation compared to others strategies ( $p < 0.01$ )(b). Volume differences data (c) highlighted that GTV volume was not preserved when using structure-guided DIR with deformable propagation as opposed to the other two strategies. The rigid registration/rigid propagation and image-guided DIR/rigid propagation strategies ensure the preservation of the volume that is propagated. No volume difference between GTVConventional and GTVPropagated is expected. Wilcoxon Signed-Rank test,  $p < 0.05$  is considered significant. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . The horizontal line in the boxplots refers to the median value, the limits of the box to the 75th and 25th percentiles, the upper whisker to the 75th percentile + 1.5 \* interquartile range, and the lower whisker to the 25th percentile - 1.5 \* interquartile range. (CB)CT: (Cone-beam) computed tomography, DIR: Deformable image registration, GTV: Gross tumour volume, ns: Not significant.

#### 4.1.3.2. Impact of radio-opaque markers on target volume propagation (step 2)

CBCT-guided stereotactic oART propagation error values (lCoM) were first determined in the cohort with markers using native planning CTs and CBCTs and then compared to values obtained when using the same images on which voxels within markers' volume had be overwritten to values similar to the surrounding liver tissue. The target volume propagation was performed with the Image-guided DIR/Rigid propagation. When using overwritten images, the median lCoM was 3.8 mm [interquartile range: 2.8 – 5.2 mm], and when using native images, the median lCoM was 4.0 mm [interquartile range: 2.5 – 5.8 mm]. These values were not significantly different ( $p = 0.06$ , paired

Wilcoxon Signed-Rank test), suggesting that there was no impact of high-density structures such as radiopaque markers on target volume propagation accuracy.

#### 4.1.3.3. *CBCT-guided stereotactic oART propagation error and PTV margin computation (step 3)*

The systematic ( $\Sigma_{PE}$ ) and random ( $\sigma_{PE}$ ) propagation errors were computed in the three dimensions for the cohorts with and without markers (total of 32 patients) and reported in Table 4.1.3. When excluding repeated CBCTs of the same sessions, the systematic and random errors were respectively 1.52 and 1.73 mm in the left-right axis, 2.16 and 1.72 mm in the antero-posterior axis, and 2.34 and 2.25 mm in the supero-inferior axis. These errors were computed on 150 CBCTs and were not significantly different compared to the values obtained when using repeated CBCTs (Table 4.1.3,  $p > 0.05$  for all the errors using the Wilcoxon Signed-Rank test).

| Axis | Systematic propagation error (mm) | Random propagation error (mm) |
|------|-----------------------------------|-------------------------------|
| LR   | 1.62                              | 1.65                          |
| AP   | 2.21                              | 1.77                          |
| SI   | 2.21                              | 2.14                          |

*Table 4.1.3: Systematic and random propagation errors of the image-guided deformable image registration with rigid GTV propagation used in the CBCT-guided stereotactic oART workflow. These values were computed for the cohort of patients with and without markers on a total of 249 cone-beam CTs. AP: Antero-posterior axis, CBCT(-STAR): Cone-beam computed*

tomography (-guided stereotactic adaptive radiotherapy), GTV: Gross tumour volume, LR: Left-right axis, PTV: Planning target volume, SI: Supero-inferior axis.

#### 4.1.3.4. Plan evaluation (step 4)

Among the 20 patients included for the plan quality evaluation, the mPTV volumes (Eq.4.1.2) were on average 69.1% larger compared to conventional PTV (Eq.4.1.1) for the same GTV per patient. Differences in margin values are described in Table 4.1.4. Plan<sub>Adaptive</sub> selection allowed better target volume coverage than Plan<sub>Non-adaptive</sub> for all the evaluated metrics (Table 4.1.5)

| Axis             | PTV    | mPTV    | Difference |
|------------------|--------|---------|------------|
| Left-right       | 4.8 mm | 9.1 mm  | + 4.3 mm   |
| Antero-posterior | 6.3 mm | 10.2 mm | + 3.9 mm   |
| Supero-inferior  | 7.2 mm | 11.8 mm | + 4.6 mm   |

Table 4.1.4: Average margin values for conventional PTV and modified PTV integrating propagation errors of the ETHOS®. (m)PTV: (modified) Planning target volume.

| Target volume coverage   | Plan <sub>Non-adaptive</sub><br>(median [P25;P75]) | Plan <sub>Adaptive</sub><br>(median [P25;P75]) | P-value          |
|--------------------------|----------------------------------------------------|------------------------------------------------|------------------|
| GTV D95% (Gy)            | 10.75 [10.54 ; 10.88]                              | 10.88 [10.69 ; 11.00]                          | <b>&lt;0.001</b> |
| GTV V100% (%)            | 99.90 [99.08 ; 100.00]                             | 100.00 [99.90 ; 100.00]                        | <b>0.005</b>     |
| GTV V110% (%)            | 87.95 [56.48 ; 95.63]                              | 92.35 [81.25 ; 96.00]                          | <b>&lt;0.001</b> |
| mPTV D95% (Gy)           | 9.71 [7.67 ; 9.91]                                 | 9.69 [8.19 ; 10.00]                            | <b>0.009</b>     |
| mPTV V100% (%)           | 90.50 [87.30 ; 93.63]                              | 93.00 [89.58 ; 95.00]                          | <b>0.008</b>     |
| Optimised mPTV V100% (%) | 93.75 [91.70 ; 95.23]                              | 95.00 [94.38 ; 95,23]                          | <b>0.044</b>     |

*Table 4.1.5: Target volume coverage comparison between Plan<sub>Non-adaptive</sub> and Plan<sub>Adaptive</sub> (n = 20 patients). The Plan<sub>Adaptive</sub> enabled better target volume coverage for all the objectives compared to the scheduled plan (Plan<sub>Non-adaptive</sub>). Wilcoxon Signed-Rank test,  $p < 0.05$  is considered significant. GTV: Gross tumour volume, mPTV: Modified planning target volume.*

There were significantly more priority 1 OAR dose constraints violations when using Plan<sub>Non-adaptive</sub> compared with Plan<sub>Adaptive</sub> (4.2 versus 0.9% of the total priority 1 OAR dose constraints among our cohort, respectively;  $p < 0.001$ ). Priority 2 constraints violations were similar between Plan<sub>Non-adaptive</sub> and Plan<sub>Adaptive</sub> (5.2 versus 6.3%, respectively;  $p = 0.999$ ) as well as for priority 3 constraints (15.2 versus 12.2%, respectively;  $p = 0.132$ , Figure 4.1.3). Heart D0.1 cm<sup>3</sup> and great vessels D0.1 cm<sup>3</sup> accounted for 39.2 and 31.6% of the

total number of priority 1 constraints violations (Table 4.1.6), respectively, whereas 94.6% of the priority 3 violations involve the chestwall. Figure 4.1.4 illustrates some differences between conventional and CBCT-guided stereotactic oART plans.

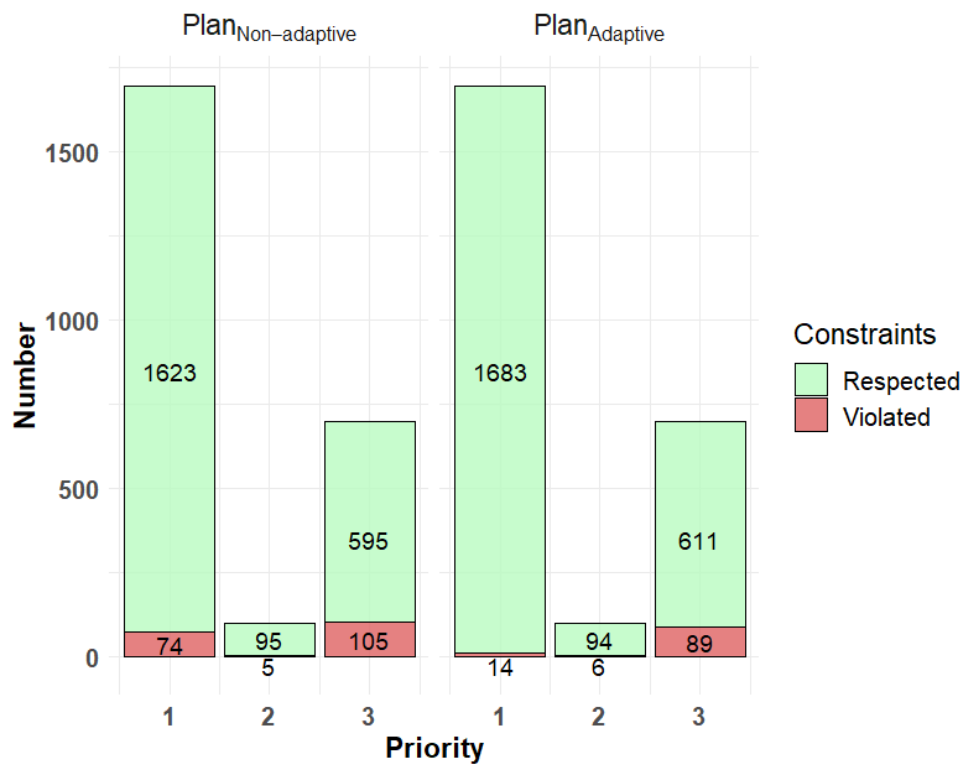


Figure 4.1.3: Organs at risk dose constraints violation comparison for each session between scheduled plans recalculated on the cone-beam computed tomography (Plan<sub>Non-adaptive</sub>) and Plan<sub>Adaptive</sub>. Priority 1 dose constraints were more likely to be violated with Plan<sub>Non-adaptive</sub> than with Plan<sub>Adaptive</sub> ( $p < 0.001$ ), no significant difference was found for other constraints. Fisher's exact test,  $p < 0.05$  is considered significant.

| PlanNon-adaptive |                                   |                     |                 | PlanAdaptive                      |                     |                 |
|------------------|-----------------------------------|---------------------|-----------------|-----------------------------------|---------------------|-----------------|
| Patients         | Goals                             | N violated sessions | Violation range | Goals                             | N violated sessions | Violation range |
| 1                | No violation                      |                     |                 | No violation                      |                     |                 |
| 2                | D0.1 cm³ Heart < 760 cGy          | 5/5                 | 782 - 1207 cGy  | No violation                      |                     |                 |
|                  | V640cGy Heart < 10 cm³            | 1/5                 | 26.9 cm³        |                                   |                     |                 |
| 3                | D0.1 cm³ Great vessels < 1060 cGy | 4/5                 | 1107 – 1149 cGy | D0.1 cm³ Great vessels < 1060 cGy | 2/5                 | 1062 – 1063 cGy |
|                  | D0.1 cm³ Heart < 760 cGy          | 2/5                 | 914 – 948 cGy   |                                   |                     |                 |
| 4                | Dmean Liver – PTV < 250 cGy       | 4/5                 | 250 – 262 cGy   | No violation                      |                     |                 |
|                  | D0.1 cm³ Stomach < 640 cGy        | 5/5                 | 703 – 1025 cGy  |                                   |                     |                 |
|                  | D10 cm³ Stomach < 360 cGy         | 2/5                 | 571 - 577 cGy   |                                   |                     |                 |
| 5                | No violation                      |                     |                 | No violation                      |                     |                 |
| 6                | No violation                      |                     |                 | No violation                      |                     |                 |
| 7                | No violation                      |                     |                 | No violation                      |                     |                 |
| 8                | No violation                      |                     |                 | No violation                      |                     |                 |
| 9                | D0.1 cm³ Heart < 760 cGy          | 2/5                 | 863 – 1015 cGy  | No violation                      |                     |                 |
|                  | Dmean Liver – PTV < 250 cGy       | 1/5                 | 242 cGy         |                                   |                     |                 |
| 10               | D0.1 cm³ Esophagus < 640 cGy      | 3/5                 | 712 – 727 cGy   | D0.1 cm³ Heart < 760 cGy          | 3/5                 | 761 – 778 cGy   |
|                  | D0.1 cm³ Heart < 760 cGy          | 5/5                 | 810 – 873 cGy   |                                   |                     |                 |
|                  | D0.1 cm³ Stomach < 640 cGy        | 3/5                 | 655 – 980 cGy   |                                   |                     |                 |

|    |                                   |     |                 |                                   |     |                 |                          |     |         |
|----|-----------------------------------|-----|-----------------|-----------------------------------|-----|-----------------|--------------------------|-----|---------|
| 11 | No violation                      |     |                 | No violation                      |     |                 |                          |     |         |
| 12 | No violation                      |     |                 | No violation                      |     |                 |                          |     |         |
| 13 | V390 cGy Esophagus < 5 cm³        | 2/5 | 5.5 - 7.1 cm³   | D0.1 cm³ Great vessels < 1060 cGy | 3/5 | 1067 -1070 cGy  |                          |     |         |
|    | D0.1 cm³ Great vessels < 1060 cGy | 5/5 | 1091 – 1123 cGy |                                   |     |                 |                          |     |         |
|    | V940 cGy Great vessels < 10 cm³   | 5/5 | 26.0 - 32.9 cm³ |                                   |     |                 |                          |     |         |
|    | D0.1 cm³ Heart < 760 cGy          | 5/5 | 762 – 1111 cGy  |                                   |     |                 |                          |     |         |
|    | V640cGy Heart < 10 cm³            | 1/5 | 10.1 cm³        |                                   |     |                 |                          |     |         |
| 14 | No violation                      |     |                 | V940 cGy Great vessels < 10 cm³   | 1/5 | 12.6 cm³        |                          |     |         |
| 15 | D0.1 cm³ Great vessels < 1060 cGy | 1/5 | 1060 cGy        | No violation                      |     |                 |                          |     |         |
| 16 | D0.1 cm³ Great vessels < 1060 cGy | 4/5 | 1062 - 1069 cGy | D0.1 cm³ Great vessels < 1060 cGy | 2/5 | 1066 - 1067 cGy |                          |     |         |
|    | D0.1 cm³ Heart < 760 cGy          | 4/5 | 787 - 1062 cGy  |                                   |     |                 | D0.1 cm³ Heart < 760 cGy | 1/5 | 773 cGy |
|    | Dmean Liver – PTV < 250 cGy       | 1/5 | 251 cGy         |                                   |     |                 |                          |     |         |
| 17 | V390 cGy Esophagus < 5 cm³        | 3/5 | 5.4 - 6.2 cm³   | D0.1 cm³ Great vessels < 1060 cGy | 3/5 | 1060 - 1075 cGy |                          |     |         |
|    | D0.1 cm³ Heart < 760 cGy          | 3/5 | 865 - 1042 cGy  |                                   |     |                 |                          |     |         |
|    | D0.1 cm³ Great vessels < 1060 cGy | 1/5 | 1099 cGy        |                                   |     |                 |                          |     |         |
| 18 | D0.1 cm³ Stomach < 640 cGy        | 1/5 | 1037 cGy        | No violation                      |     |                 |                          |     |         |
| 19 | D0.1 cm³ Duodenum < 640 cGy       | 4/5 | 674 - 698 cGy   | No violation                      |     |                 |                          |     |         |
|    | D0.1 cm³ Large bowel < 760 cGy    | 2/5 | 764 - 773 cGy   |                                   |     |                 |                          |     |         |
| 20 | No violation                      |     |                 | No violation                      |     |                 |                          |     |         |



*Table 4.1.6: Priority 1 and 2 organ-at-risk constraint violations on an individual patient basis for each individual session. Organ-at-risk dose constraints are expressed as the dose objective per individual session. PTV: Planning target volume.*

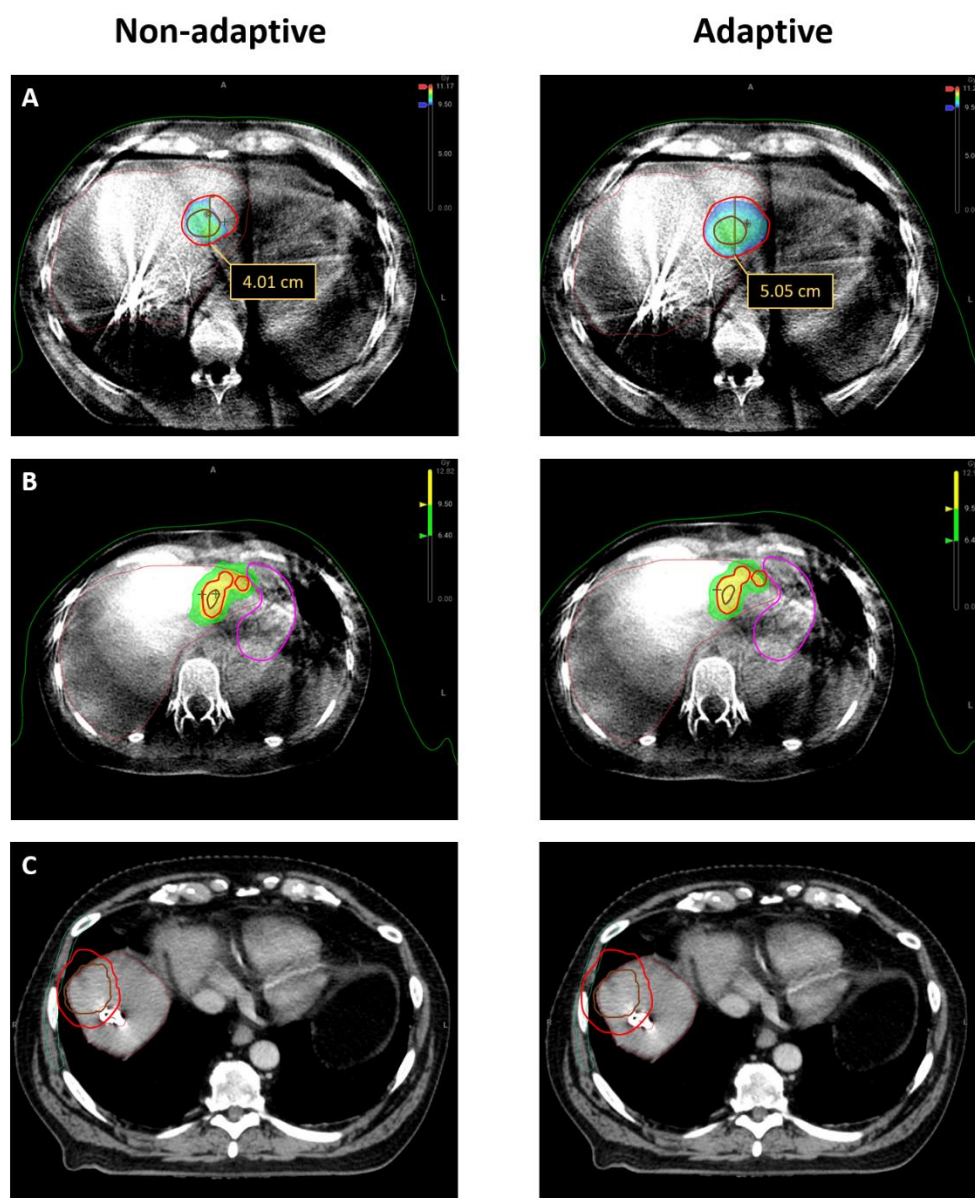


Figure 4.1.4: Illustration of the impact of CBCT-guided stereotactic oART . In case A, daily adaptation enabled better dose coverage of the PTV (red line), despite the fact that the mPTV used in the CBCT-guided stereotactic oART workflow (5.0 cm diameter) is bigger than the scheduled PTV (4.0 cm diameter). The dose shown in this image is 95% of the prescription dose. Daily

*adaptation allowed to spare organs at risk that are in the vicinity of the tumour. In case (B), using the non-adaptive plan would have led to the maximum dose (green isodose line) tolerated by the stomach (pink line) being exceeded. However, using the PlanAdaptive allowed to protect the stomach from excessive doses. However, in some patients (C), the larger mPTV (red line) used in the CBCT-guided stereotactic oART workflow could overlap with other organs-at-risk, in most of cases the chestwall, and potentially result in a violation of their dose constraints. CBCT-guided stereotactic oART : Cone-beam computed tomography-guided stereotactic adaptive radiotherapy, mPTV: Modified planning target volume.*

#### **4.1.3.5. Time results**

The mean time of CBCT-guided stereotactic oART sessions was 15.1 minutes from CBCT importation to plan approval. It ranged from 9.9 to 23.0 minutes.

#### **4.1.4. Discussion**

In this study, we demonstrate that markerless liver SABR is feasible using a CBCT-based online-adaptive radiotherapy workflow, provided the PTV margin is increased in order to compensate for propagation errors (Eq.4.1.2). Indeed, among the 1697 priority 1 OAR dose constraints that were evaluated, only 14 were violated in our cohort of 20 patients. These violations mainly involved the heart and great vessels (aorta and inferior vena cava). This could lead to a potential increased risk of long-term cardiac toxicity or, in the case of great vessel overdosage, to radiation-induced aneurysm or bleeding, which are rarely described in liver SABR [496, 497]. However, in most cases, these priority 1 OAR dose constraint violations

would have been predicted by the radiation oncologist at the time of treatment planning by considering the anatomical relationship between OARs and target volumes. Some treatment parameters would consequently have been adjusted to alleviate the risk of radio-induced complications. For example, here, we used a standard dose of 5 x 10 Gy for all our plans evaluation, but in clinical routine, some more fractionated schemes exist and could have been used instead, with a similar local control rate but a reduction of the total biological equivalent dose delivered to OARs [498, 499]. Another option could be to modify the prioritisation weight according to these critical OARs to prevent potential overdosage. To note, constraints for serial organs were accepted as soon as the D0.1 cm<sup>3</sup> was respected in the priority 1 level, as usually accepted in usually daily online adaptive radiotherapy workflows.

Liver SABR could obviously benefit from MRI-guided online-adaptive radiotherapy that allows for direct visualisation of the hepatic lesion without radio-opaque markers [183-185]. Rosenberg et al. reported a prospective study of 26 liver SABR patients, in whom a median dose of 5 x 10 Gy was delivered by an MRI-guided linac [183]. The local progression-free rate reached 80.4% after a median follow-up time of 21.2 months. The treatment was well tolerated, with a low grade 3 toxicity rate (n = 2) and no grade 4-5 toxicity occurrence. However, this technique has several drawbacks. First of all, MRI-guided radiotherapy requires an expensive infrastructure as well as a major involvement of very specialised staff, which restrains its accessibility [184, 187]. Second, MRI comes with some image distortion and artefacts that may induce inaccuracies, leading consequently to geographic misses

[183, 184, 187]. Third, contraindications to this treatment exist for patients with MRI-incompatible medical devices, such as pacemakers, or sometimes for patients with a high degree of anxiety or claustrophobia [186]. Fourth, MRI-guided treatment duration is longer than treatment on conventional linacs and could potentially induce patient discomfort [183, 185, 187]. In the Rosenberg et al. study, the reported treatment duration was 40-60 minutes [183]. CBCT-guided stereotactic oART using the ETHOS may share the same potential contraindication with MRI-guided linac in patients with claustrophobia, probably in a lesser degree since the tunnel effect is not described as for MRI devices or Linacs and no specific antennas are required to improve the signal. Regarding the session duration, our results could not be compared with those reported for MRI-guided RT because we do not integrate the time for patient setup, CBCT acquisition, x-ray delivery, and patient discharge, as all our CBCT-guided stereotactic oART sessions were performed in a virtual environment. We report an average virtual session duration from the CBCT importation to the plan approval of 15.1 minutes. Due to the retrospective and *in-silico* design of this study, these results should be considered carefully and do not perfectly reflect the clinical conditions. These values are lower than those reported in the literature for upper abdominal locations. Schiff et al. published two *in-silico* feasibility analyses of CBCT-guided stereotactic oART for the treatment of locally advanced pancreatic cancer and abdominal metastases. The average duration with similar timepoints was 36.3 and 22.9 minutes for these two locations, respectively [313, 314]. This difference in time results could be explained because only a single well-trained radiation oncologist performed

all the virtual sessions, avoiding the learning curve requested to become familiar with the software [313, 314].

As demonstrated here, CBCT-guided stereotactic oART is not only feasible but is, in addition, an effective strategy to compensate for interfraction anatomical variations, and reduce OAR dose constraint violations. The daily-adapted plans allowed us to reduce by 42.0% the total number of OARs whose dose constraints would have been violated by the systematic use of scheduled (non-adapted) plans. This is even more important to consider for the priority 1 OAR dose constraints, with a 79.7% reduction in the violation rate among the whole population thanks to adaptation. In most of the cases, the target volume coverage improved when using adaptation. On an individual basis, the violation of priority 1 and 2 OAR dose constraints occurred in 11/20 patients when using non-adapted plans. Meanwhile, the adapted plans enabled the reduction of the number of constraint violations in each of these 11 patients as a result of only 6 patients having priority 1 and 2 OAR dose constraint violations when using these adapted plans. Therefore, we believed that CBCT-guided stereotactic oART could be beneficial for a considerable proportion of patients. Moreover, daily planning allowed for more important GTV dose escalation (as recommended in SABR), compared to non-adapted plan. These data are concordant with those of CBCT-guided stereotactic oART treatments delivered for pelvic locations as well as for upper abdominal locations [192, 313, 314, 500]. In their *in-silico* study of Schiff et al. evaluating the feasibility of CBCT-guided stereotactic oART for abdominal oligometastatic lesions, the use of non-adapted plans would have potentially led to 53 OAR constraints violations

versus only 2 cases when applying daily adapted plans. Also, they could achieve an increase of 7.8% in the median PTV optimisation coverage when using adaptive RT instead of non-adaptive RT [314]. Those results were confirmed in their second study evaluating CBCT-guided stereotactic oART for locally advanced pancreatic cancer [313]. Their team has recently reported the first successful CBCT-guided stereotactic oART treatment for pain palliation of a pancreatic mass of 61-year-old women with metastatic pancreas cancer [501].

The method used to determine the expected GTV position on the CBCT is the major limitation of this study. The GTV<sub>Conventional</sub> definition is based on the conventional image-guided RT procedure routinely used in non-adaptive strategies: manual rigid registration and rigid GTV propagation from the planning CT to the CBCT of the day. This implies some important limitations. Our GTV<sub>Conventional</sub> could therefore not be considered a “GTV groundtruth”. To get the groundtruth position of the GTV, the most accurate solution would have been to obtain daily MRI and perform a daily delineation of the GTV on the MRI. As the ETHOS’ online-adaptive treatment planning system is based on CT or CBCT images, only the creation of a synthetic (CB)CT based on these MRI images could be considered for use in the workflow proposed here [502, 503]. However, due to the retrospective design of this study, daily MRI images are not available. In addition, even if radio-opaque markers around the tumour are a better surrogate for the liver tumour position compared to anatomical landmarks (vertebrae and diaphragm dome), they do not perfectly predict the position of the tumour. This uncertainty becomes even greater as the tumour–marker distance increases [181, 483].

To handle this issue, patients with radio-opaque markers located too far from the tumour were excluded from our cohort. Also, the relationship between radio-opaque markers and tumour position has to remain stable from the planning phase to the end of the radiotherapy treatment to be considered a valid surrogate. However, interfraction variation in this correlation exists, illustrating the limitations of marker-guided image-guided RT for liver SABR [483, 504]. Based on MRI-guided online-adaptive radiotherapy reports, we considered that the shape and volume of the liver tumour remained stable for the entire treatment course [505-507]. However, Xu et al. showed a trend towards slightly increased volume (+ 1.9% and 1.0% in the second and third fractions, respectively) from the first to the last session, possibly due to radiation-induced oedema [232]. Both our conventional clinical and CBCT-guided stereotactic oART workflows do not handle these uncertainties, which should be considered when determining the PTV margin. It could be done, for example, by integrating it as a baseline uncertainty component in the van Herk's recipe or, in the case of interfraction time trends, by applying a PTV margin determination methodology based on Monte Carlo simulations for the establishment of residual errors similar to that published by Gizynska et al [508]. However, the accurate quantification of these uncertainties will also require the GTV groundtruth determination on daily MRI, which is not possible since we do not have these images in this retrospective study. Well aware of these uncertainties associated with our definition of the GTV<sub>Conventional</sub>, we never meant to use CBCT-guided stereotactic oART to improve the GTV propagation accuracy compared to the conventional workflow. We present



here a method for obtaining GTV propagation accuracy results comparable to those of the conventional workflow while avoiding the need for radio-opaque markers.

This *in-silico* study has several other drawbacks and limitations, particularly due to its retrospective design. Indeed, the majority of the CBCTs (244/249) used in our analysis were obtained from different devices of the same manufacturer but with poorer image quality than those provided by the new ETHOS CBCT imager (only 5 CBCTs were performed with the Halcyon®, which has the same CBCT imager as the ETHOS). It impacts the OAR delineation accuracy, and it may lead to a poorer performance of the structure-guided DIR algorithm. Furthermore, these 3D-CBCTs used in the CBCT-guided stereotactic oART workflow were co-registered with an average planning CT that was acquired following a different modality. This can impact both the structure-guided and image-guided DIR algorithms and, therefore, reduce the accuracy of target volume propagation. Our mPTV margin (Eq.4.1.2) could, consequently, be probably overestimated. As a result, the target volume propagation and mPTV margin data presented here can be interpreted as a “worst-case scenario” of what might happen in clinical practice. Also, to increase the number of images for our target volume propagation analysis, we included repeated CBCTs from the same treatment day. That could potentially impact our results, but when excluding these repeated CBCTs, the propagation error values were not significantly different than those calculated when repeated CBCTs were included. These repeated CBCTs were not included in the dosimetry analysis in order to limit the risk of bias. The implementation of the liver CBCT-guided stereotactic

oART workflow with a new generation CBCT imager as well as a respiratory motion-management strategies such as abdominal compression or breath-holding (BH) could eventually improve the CBCT quality and, thus, lead to a mPTV margin reduction [507, 509]. Further investigations using those techniques are requested to refine our observations.

This study is a first step in performing markerless liver CT-STAR. Our model can be improved by a precise determination of the real GTV position on CBCT to evaluate the accuracy of the GTV<sub>Propagated</sub>. Here again, further prospective investigations are needed. However, these results provide an opportunity for patients with anatomical considerations or comorbidities that contraindicate radio-opaque marker implantation. Moreover, this study gives insights about the dosimetric advantages of performing CBCT-guided stereotactic oART instead of non-adaptive RT in the presence of radiopaque markers. Compared with the markerless technique, PTV could be reduced, and, therefore, the daily adaptive plan computation could allow better sparing of OARs and better coverage of target volumes.

#### **4.1.5. Conclusion**

This retrospective *in-silico* study demonstrates that markerless liver SABR is feasible when using a CT-based online-adaptive radiotherapy workflow. The propagation error is minimal when applying the Image-guided DIR/Rigid propagation strategy for target volume propagation and is not influenced by the presence of the radio-opaque markers. While the incorporation of this propagation error in the PTV margin results in a larger mPTV volume, daily

dosimetry adaptation allowed us to meet the OAR dose constraints and target volume coverage objectives in most cases.

## **4.2. Mechanically assisted non-invasive ventilation for liver SABR: Improve CBCT, treat more accurately**

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## Highlights

- Mechanically assisted non-invasive ventilation is used for CBCT-guided liver SABR.
- CBCT image quality is better with MANIV compared to free-breathing.
- MANIV facilitates image-guided RT and reduces its interobserver variability.
- OARs auto-segmentation is more accurate on MANIV CBCTs.
- Time dedicated to correction of auto-segmented OARs is reduced with MANIV CBCTs.

## **Abstract**

### *Background and purpose*

Cone-beam computed tomography (CBCT) for image-guided radiotherapy during liver stereotactic ablative radiotherapy (SABR) is degraded by respiratory motion artefacts, potentially jeopardising treatment accuracy. Mechanically assisted non-invasive ventilation-induced breath-hold (MANIV-BH) can reduce these artefacts. This study compares MANIV-BH and free-breathing CBCTs regarding image quality, image-guided RT variability, automatic registration accuracy, and deep-learning auto-segmentation performance.

### *Materials and methods*

Liver SABR CBCTs were presented blindly to 14 operators: 25 patients with free-breathing and 25 with MANIV-BH. They rated CBCT quality and image-guided RT ease (rigid registration with planning CT). Interoperator image-guided RT variability was compared between free-breathing and MANIV-BH. Automatic gross tumour volume (GTV) mapping accuracy was compared using automatic rigid registration and image-guided deformable registration. Deep-learning organ-at-risk (OAR) auto-segmentation was rated by an operator, who recorded the time dedicated for manual correction of these volumes.

### *Results*

MANIV-BH significantly improved CBCT image quality ("Excellent"/"Good": 83.4% versus 25.4% with free-breathing,  $p < 0.001$ ), facilitated image-guided RT ("Very easy"/"Easy": 68.0% versus 38.9% with free-breathing,  $p < 0.001$ ),

and reduced image-guided RT variability, particularly for trained operators (overall variability of 3.2mm versus 4.6mm with free-breathing,  $p=0.010$ ). MANIV-BH improved deep-learning auto-segmentation performance (80.0% rated "Excellent"/"Good" versus 4.0% with free-breathing,  $p<0.001$ ), and reduced median manual correction time by 54.2% compared to free-breathing ( $p<0.001$ ). However, automatic GTV mapping accuracy was not significantly different between MANIV-BH and free-breathing.

### *Conclusion*

In liver SABR, MANIV-BH significantly improves CBCT quality, reduces interoperator image-guided RT variability, and enhances OAR auto-segmentation. Beyond being safe and effective for respiratory motion mitigation, MANIV increases accuracy during treatment delivery, although its implementation requires resources.

#### **4.2.1. Introduction**

Liver tumours, including primary malignancies and metastases, represent a significant global health issue due to rising incidence and persistently high mortality rates [1, 510, 511]. Liver stereotactic ablative radiotherapy (SABR) is increasingly used for managing both primary and secondary tumours [39, 480, 512]. In curative treatment of hepatocellular carcinoma and colorectal liver metastases, SABR can serve as a bridging therapy to enhance local control before transplantation [39, 480]. For patients ineligible for transplantation or surgery, SABR offers a safe and effective alternative to radiofrequency ablation and transarterial chemoembolization, as recommended by ESMO and EASL guidelines [480, 513-515]. SABR achieves up to 82% 5-year local control in hepatocellular carcinoma [512]. However, accurate image-guided radiotherapy and effective respiratory motion management remain critical challenges for accurate targeting and sparing of organs-at-risk (OARs).

Accurate image-guided RT is complex due to the similar density of liver tumours and surrounding tissue on cone-beam computed tomography (CBCT), with image quality often compromised by respiratory artefacts [516]. Strategies to overcome this include magnetic resonance (MRI)-guided RT, which enables direct and real-time visualisation of liver tumours and adjacent OARs during daily image-guided RT [183-185]. Another option is the percutaneous implantation of radiopaque markers as surrogates for tumour position during CBCT-guided image-guided RT [180]. Recently, CBCT-guided online-adaptive RT (oART) has been evaluated for liver SABR [278]. In oART,



OARs are automatically delineated, and target volumes are automatically propagated from planning CT to daily CBCT using a deformable registration. While adaptive planning can improve OARs sparing and target volume coverage, automatic propagation of target volumes introduces uncertainties that should be integrated in planning target volume (PTV) margins. In the context of liver SABR, these uncertainties results in PTV margins that are larger in oART compared to non-ART [278]. In practice, many centres still avoid such complex, resource-intensive techniques and rely on broader margins despite their associated limitations.

Respiratory motion strategies include passive (e.g., internal target volume, mid-position, abdominal compression) or active techniques (e.g., breath-hold, respiratory gating, tumour tracking) [172]. Passive techniques are widely used due to ease of implementation. Active strategies, though more complex and resource demanding, aim for greater accuracy with reduced OAR irradiation. Among them, mechanically-assisted non-invasive ventilation (MANIV) for breath-hold (BH) is particularly promising. MANIV-BH enables reproducible deep inspiration breath-holds, reducing breathing-related motion [176-178]. MRI studies have confirmed its feasibility and ability to induce stable apneas [176-179]. MANIV-BH is safe for liver SABR and, compared to other techniques, may reduce PTV margin [178]. Moreover, immobilisation of the thorax and upper abdomen during MANIV-BH could be can minimise respiratory artefacts on CBCT and thereby improving image-guided RT accuracy.

This study aims to compare CBCTs acquired with MANIV-BH versus free-breathing (free-breathing) CBCTs in liver SABR patients to assess their impact on image quality and treatment precision. The impact of MANIV-BH was assessed through a multi-step approach for both non-adaptive RT (Non-ART) and oART strategies, progressing from basic to advanced metrics: (1) Image quality assessment; (2) image-guided RT variability; (3) Automatic registration accuracy; and (4) deep-learning auto-segmentation performance.

#### **4.2.2. Materials and Methods**

This monocentric *in silico* study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Cliniques Universitaires Saint-Luc.

##### **4.2.2.1. Patients**

Patient were retrospectively included between January 2021 and July 2024 and allocated into two groups based on the respiratory motion management strategy: MANIV-BH versus free-breathing. The sample size was fixed a priori at 25 patients per group. Inclusion criteria were as follows: (1) adult patients (> 18 years old); (2) patients treated with liver SABR or RT for stomach, adrenal or pancreas tumour that included an identifiable structure within the liver (e.g., cyst, hemangioma or tumour) on planning CT ; (3) patients treated on linear accelerators sharing identical CBCT acquisition device

(Halcyon® and Ethos®, Varian, a Siemens Healthineers Company, Palo Alto, CA).

#### *4.2.2.2. RT treatment and CBCT acquisition*

For free-breathing patients, a 4D planning CT with audio-coaching and abdominal compression was acquired, while for MANIV-BH patients a 3D planning CT was used. All CTs were acquired in supine with intravenous iodine contrast injection. RT planning was performed using the Raystation Planning system (clinical versions 12A, RaySearch Laboratories, Stockholm, Sweden). Prescription doses and target volume delineation were adapted based on the RT intent. Daily CBCTs were acquired using the “pelvis fast” (acquisition time: 21.2 seconds) or “pelvis large fast” (acquisition time: 25 seconds) modes on both linear accelerators for free-breathing and MANIV-BH patients (slice thickness: 2 mm, Figure 4.2.1). image-guided RT was performed using a tumour-based rigid registration between the planning CT and the CBCTs. For MANIV-BH patients, multiple CBCTs were acquired to assess the BH reproducibility (internal image-guided RT protocol).

MANIV-BH procedure has been described in detail in previous studies (Figure 4.2.1A) [176, 178]. Briefly, prior to the planning CT acquisition, patients were trained to familiarise with the procedure. The ventilatory parameters were individually determined by a physiotherapist. Following connexion to the mechanical ventilator (Bellavista 1000, Vyair Medical, Mettawa, Illinois, USA), pre-oxygenation was initiated (Fi60%) while patients continued to breathe without constraint. To induce apnea, the adaptive

pressure release ventilation mode was activated. This mode consists of two phases: (1) Inhale and apnea phase: a high constant pressure (range: 15-19 mmHg) was sustained for 30 seconds; (2) Exhale phase: a zero-pressure state (range: 4.1 – 5.1 seconds) allowed the patient to breathe out. This cycle (1+2) was repeated throughout the RT session. The radiation therapy technologist (radiotherapy technologist) manually activated the beam during the apneas and interrupted the beam just before the second phase, based on the ventilator's pressure curve, which was displayed in the control room.

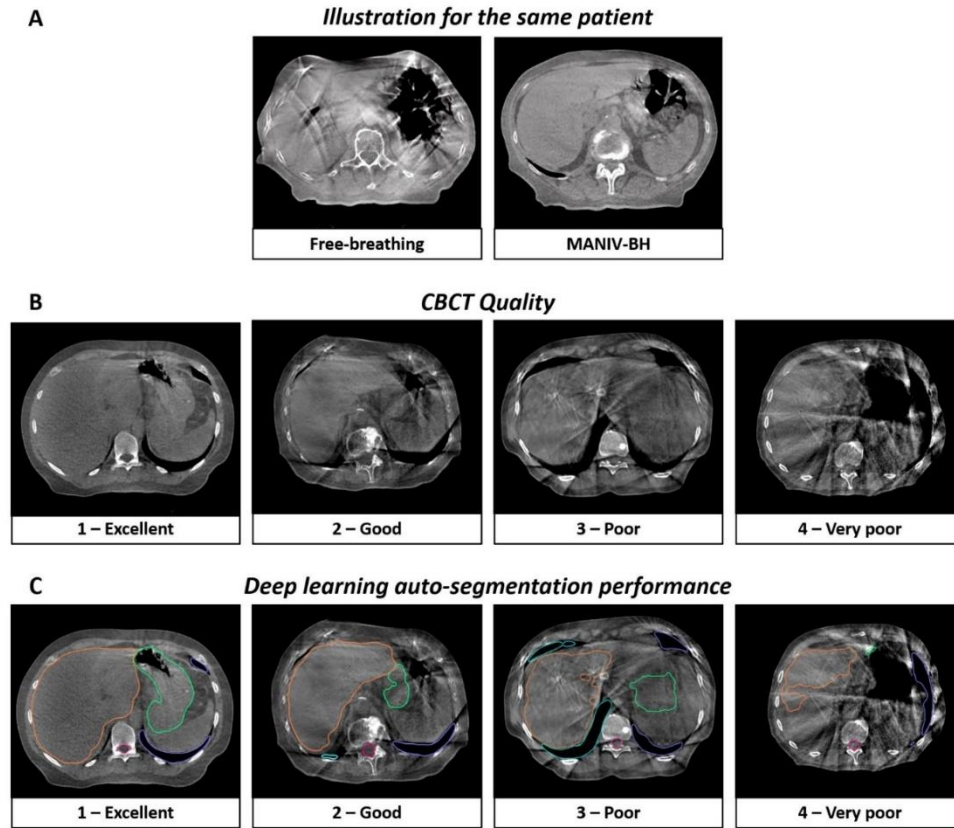


Figure 4.2.1: Illustrations of (A) CBCTs acquired with free-breathing and with MANIV-BH for the same patient, (B) different CBCT quality and (C) performance of auto-segmentation by a deep learning algorithm. CBCT: Cone-beam computed tomography, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath-old.

For this study, the planning CT with the gross tumour volume (GTV) of the liver and the first CBCT of each of the 50 included patients were used. In cases of pancreatic, adrenal or stomach cancer treatment, a “virtual” liver GTV was retrospectively delineated based on a pre-existing identifiable

structure selected within the liver. All CBCTs were anonymised and presented randomly and blindly to the participating operators.

#### *4.2.2.3. Image quality assessment*

Nine radiation oncologists and five radiotherapy technologists assessed the quality of CBCT images using a four-point scoring scale developed for this study: 1 – Excellent, 2 – Good, 3 – Poor, and 4 – Very poor.

#### *4.2.2.4. Image-guided RT variability*

The same operators then performed a GTV-based rigid registration between the planning CT and the CBCTs using translation only, mimicking the actual clinical practice in the institution. They rated the ease of performing this matching using another four-point scoring scale developed for this study: 1 – Very easy, 2 – Easy, 3 – Hard, and 4 – Very hard. For each CBCT, the standard deviation of the registration vector across operators was calculated in right-left, antero-posterior and supero-inferior directions. The overall variability was quantified using the total standard deviation of the registration, defined as the square root of the sum of the variances in each dimension. Among the radiation oncologists, five had over four years of experience and were considered fully trained in liver SABR image-guided RT. A separate analysis of this subgroup was conducted to provide a more accurate reflection of clinical practice.

#### 4.2.2.5. *Automatic GTV mapping*

Two automatic GTV mappings from the planning CT to the CBCT were analysed, corresponding to conventional Non-ART and oART strategies.

For the Non-ART strategy, an automatic rigid registration using only translations focused on the GTV region of interest was performed between the planning CT and the CBCT using the Raystation® Planning system. The GTV from the planning CT was then transferred to the CBCT (GTV<sub>Non-ART</sub>).

For the oART strategy, virtual oART sessions were conducted using the Ethos® emulator. This emulator includes both the oART treatment planning system and the online adaptive radiotherapy software, enabling virtual delivery of oART sessions using planning CT and CBCT images retrieved from a database. During these virtual sessions, the GTV was automatically propagated (GTV<sub>oART</sub>) onto the CBCT via image-guided deformable registration with rigid volume propagation (research version). However, the emulator was no longer provided to our department by the vendor at the time of this analysis, and results were only available for the first 20 MANIV-BH patients. These results were compared to a previously published cohort of 21 free-breathing patients [278].

The methodology used for propagation accuracy assessment was detailed in prior work [278]. Briefly, the propagated GTVs (GTV<sub>Non-ART</sub> or GTV<sub>oART</sub>) were compared to the reference GTV positions on the daily CBCT (GTV<sub>Groundtruth</sub>). This GTV<sub>Groundtruth</sub> was defined as the median GTV position among trained operators during the image-guided RT variability analysis. GTV positions were compared using two metrics: (1) the Euclidean distance between the

centres of mass of the propagated GTV and the GTV<sub>Groundtruth</sub> (CoM), and the Hausdorff 95% distance, which is the greatest distance between these volumes among their 95% closest voxels.

#### *4.2.2.6. Deep-learning auto-segmentation performance*

As for the previous analysis on “Automatic GTV mapping”, the availability of the ETHOS<sup>®</sup> emulator was discontinued for this analysis. Therefore, the deep-learning segmentation model integrated into the institutional treatment planning system (Raystation<sup>®</sup>) was used to delineate abdominal OAR volumes on each CBCT. A single RO assessed the segmentation quality for both overall and individual OARs using a four-point scoring scale: 1 – Excellent, 2 – Good, 3 – Poor, and 4 – Very poor. Subsequently, the RO manually corrected the segmented volumes on transversal CBCT slices over a 10 cm height, with the most cranial part of the L1 vertebral body serving as the central reference slice. The list of OARs included both lungs, the heart, the spinal canal, the liver, the oesophagus, the stomach, and both kidneys. The time required for manual corrections was recorded as an additional measure of auto-segmentation performance.

#### *4.2.2.7. Statistical analysis*

The median value and interquartile range (interquartile range) were used to describe quantitative data. Comparisons between free-breathing and MANIV-BH groups were conducted using the Chi-square test for qualitative



variables and the Wilcoxon signed-rank test for quantitative variables. A significance threshold was set at a  $p < 0.05$ . For multiple comparisons, p-values were adjusted using the Benjamini-Hochberg method to control for false discovery rates. All statistical analyses were performed using RStudio (R version 4.2.1) with the "tidyverse" package.

#### **4.2.3. Results**

The characteristics of the included patients are described in Table 4.2.1. For details on the free-breathing population used in the "oART GTV propagation" analysis, refer to Table A9.1.

|                                                                  | Free-breathing patients | MANIV-BH patients |
|------------------------------------------------------------------|-------------------------|-------------------|
| <b>N</b>                                                         | 25                      | 25                |
| <b>Gender</b>                                                    |                         |                   |
| Male                                                             | 15                      | 15                |
| Female                                                           | 10                      | 10                |
| <b>Age (median [interquartile range])</b>                        | 72 (66 – 79)            | 65 (56 – 72)      |
| <b>Radiotherapy intent</b>                                       |                         |                   |
| <i>Primary disease</i>                                           |                         |                   |
| Hepatocarcinoma                                                  | 12                      | 9                 |
| Gastric lymphoma*                                                | 0                       | 6                 |
| Pancreatic adenocarcinoma*                                       | 2                       | 5                 |
| <i>Liver metastasis from</i>                                     |                         |                   |
| Colorectal adenocarcinoma                                        | 7                       | 1                 |
| Non-small cell lung cancer                                       | 2                       | 2                 |
| Breast cancer                                                    | 1                       | 0                 |
| Renal cell carcinoma                                             | 1                       | 0                 |
| Cervical cancer                                                  | 0                       | 1                 |
| Gastroesophageal junction cancer                                 | 0                       | 1                 |
| <b>Tumour location**</b>                                         |                         |                   |
| Segment II                                                       | 2                       | 4                 |
| Segment III                                                      | 2                       | 2                 |
| Segment IV                                                       | 3                       | 7                 |
| Segment V                                                        | 5                       | 4                 |
| Segment VI                                                       | 6                       | 4                 |
| Segment VII                                                      | 6                       | 4                 |
| Segment VIII                                                     | 6                       | 6                 |
| <b>GTV volume (cm<sup>3</sup>, median [interquartile range])</b> | 9.2 (5.2 – 12.4)        | 8.8 (3.5 – 26.2)  |

Table 4.2.1: Characteristics of the patients. \*Patients for whom a “virtual” liver GTV was delineated based on an identifiable structure within the liver.

*\*\*Some patients had tumour involving multiple hepatic segments. GTV: Gross tumour volume, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold.*

#### **4.2.3.1. Image quality assessment**

Operator evaluations revealed that the quality of MANIV-BH CBCTs was significantly better than that of free-breathing CBCTs. Specifically, 83.4% of MANIV-BH CBCTs were rated as "Excellent" or "Good," compared to only 25.4% of free-breathing CBCTs ( $p < 0.001$ , Figure 4.2.1B and Figure 4.2.2A).

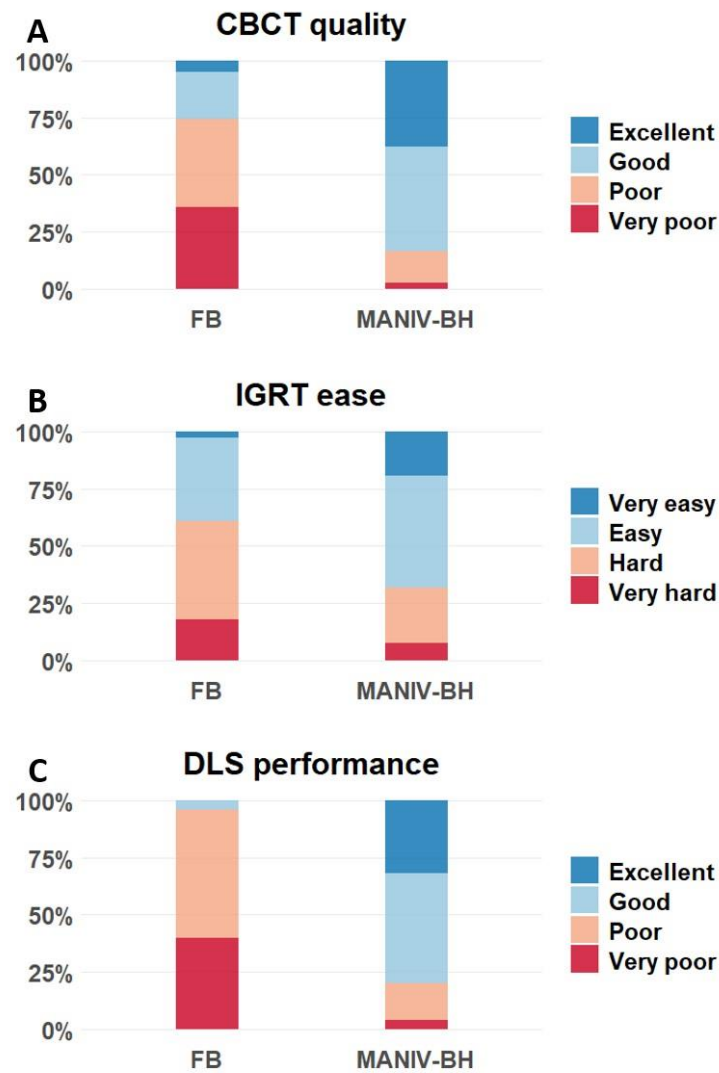


Figure 4.2.2: Comparison between CBCTs acquired using free-breathing or MANIV-BH by four-point scoring scales for (A) the quality of the CBCT, (B) the ease to perform an image-guided RT registration between the planning CT and the CBCT, and (C) the performance of the auto-segmentation of organs-at-risk using a deep learning algorithm. CBCT: Cone-beam computed tomography, DLS: Deep-learning segmentation, FB: Free breathing, IGRT: Image-guided radiotherapy, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold.

#### 4.2.3.2. *Image-guided RT variability*

The ease of registration between the planning CT and the CBCT was significantly greater with MANIV-BH CBCTs (Very easy or easy: 68.0%) compared to free-breathing CBCTs (Very easy or easy: 38.9%,  $p < 0.001$ , Figure 4.2.2B). When considering all operators, the image-guided RT variability was similar between free-breathing and MANIV-BH CBCTs in the left-right axis (median: 2.5 mm versus 2.3 mm,  $p = 0.45$ ), the antero-posterior axis (2.6 mm versus 3.5 mm,  $p = 0.062$ ), and overall (median: 5.8 mm versus 5.5mm,  $p = 0.30$ ). However, in the supero-inferior direction, the image-guided RT variability was significantly reduced with MANIV-BH CBCTs (median: 3.0 mm) compared to free-breathing CBCTs (median: 3.3 mm,  $p = 0.043$ , Figure 4.2.3A). Focusing on data from the five radiation oncologists trained in liver SABR image-guided RT, the image-guided RT variability was lower with MANIV-BH compared to free-breathing CBCTs in the left-right direction (median: 1.6 mm versus 2.1 mm,  $p = 0.026$ ), the supero-inferior direction (median: 2.2 mm versus 3.0 mm,  $p = 0.004$ ), and overall (median: 3.2 mm versus 4.6 mm,  $p = 0.010$ ), but not in the antero-posterior direction (median: 1.6 mm versus 1.7 mm,  $p = 0.67$ , Figure 4.2.3B). A comparison between trained and non-trained operators revealed significantly higher image-guided RT variability for non-trained operators in all directions when using MANIV-BH CBCTs ( $p < 0.05$ ). In contrast, for free-breathing CBCTs, this difference was only observed in the anteroposterior direction (Figure A9.1).

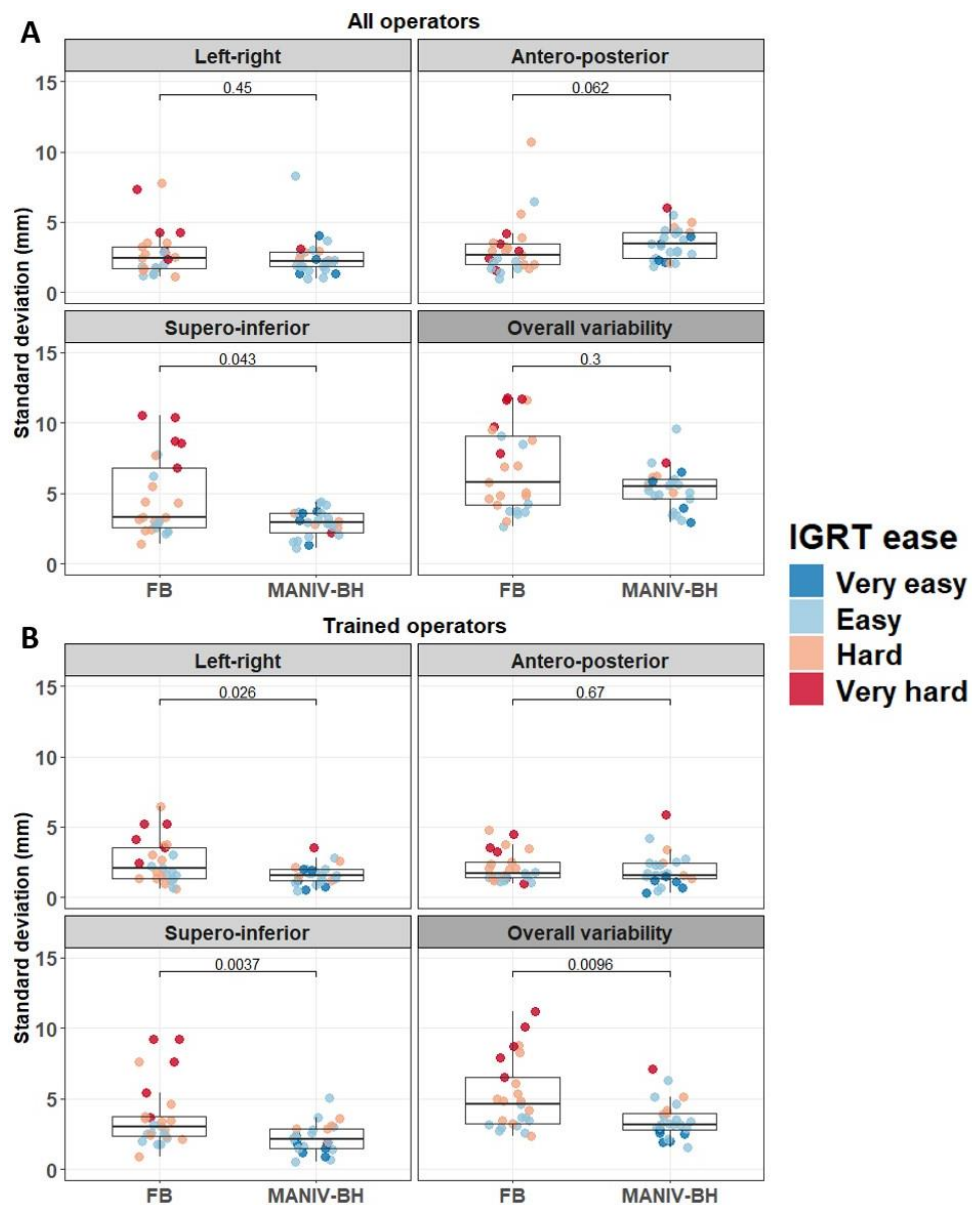


Figure 4.2.3: Comparison of the image-guided RT registration variability between CBCTs acquired using free-breathing or MANIV-BH. Each dot represents one CBCT for which the standard deviation of the registration vector was computed among all operators. (A) In the first analysis, the image-guided RT performed the nine radiation oncologists and the five

*radiotherapy technologists was considered, (B) while in the second only the five radiation oncologists a sufficient training in liver SABR image-guided RT were included. The colours of the points, each representing a CBCT, were determined based on the most frequent image-guided RT ease score reported by the operators. CBCT: Cone-beam computed tomography, FB: Free breathing, IGRT: Image-guided radiotherapy, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold, RO: Radiation oncologist, radiotherapy technologist: Radiation therapist, SABR: Stereotactic ablative radiotherapy.*

#### **4.2.3.3. Automatic GTV mapping**

For the automatic rigid registration, the median distance between the CoM of GTV<sub>Non-ART</sub> and GTV<sub>Groundtruth</sub> was 5.7 mm (interquartile range: 3.7 - 9.7) and 3.6 mm (interquartile range: 2.7 - 10.7) for free-breathing and MANIV-BH respectively ( $p = 0.20$ , Figure 4.2.4A). The median Hausdorff 95% distance between these two structures was 5.6 mm (interquartile range: 3.7 – 8.8) for free-breathing and 4.0 mm (interquartile range: 2.3 – 8.4) for MANIV-BH ( $p = 0.18$ , Figure 4.2.4B).

Using oART GTV propagation based on an image-guided deformable registration, the median CoM distance between GTV<sub>oART</sub> and GTV<sub>Groundtruth</sub> was 4.7 mm (interquartile range: 3.4 – 6.7) for free-breathing and 4.4 mm (interquartile range: 2.6 – 5.9) for MANIV-BH ( $p = 0.23$ , Figure 4.2.4C). The median Hausdorff 95% was 4.2 mm (interquartile range: 2.5 – 5.7) for free-breathing and 3.8 mm (interquartile range: 2.5 – 5.3) for MANIV-BH ( $p = 0.69$ , Figure 4.2.4D).

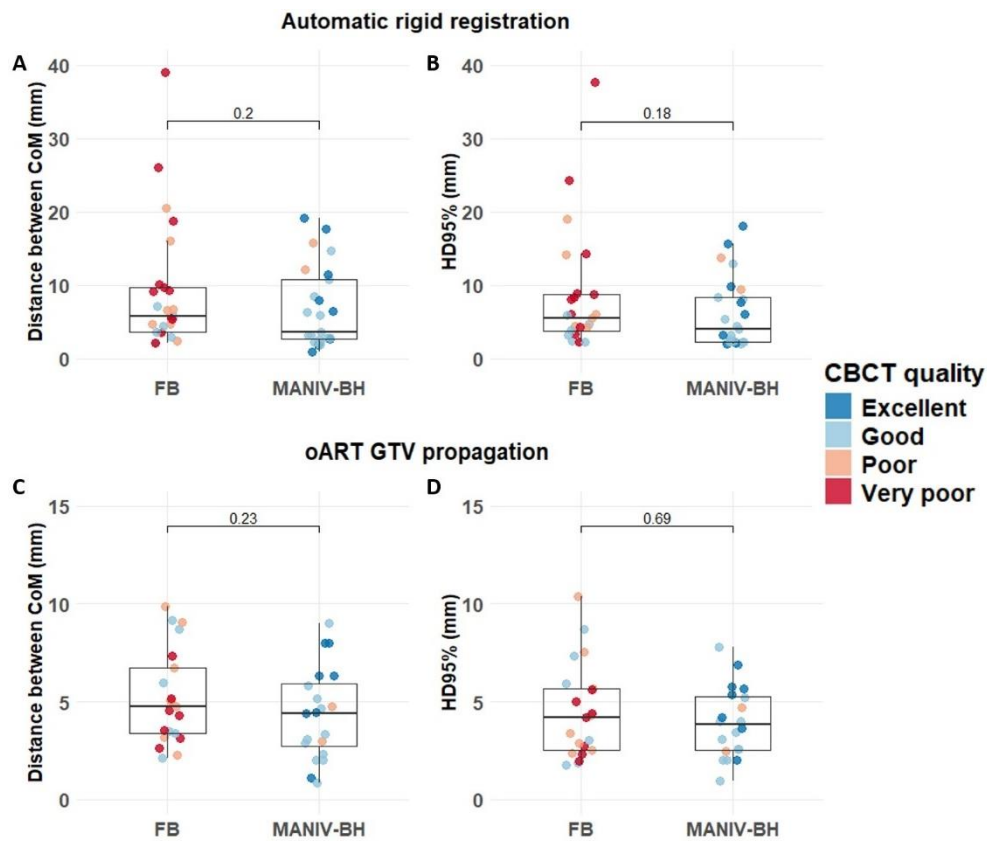


Figure 4.2.4: Accuracy of the automatic GTV mapping compared between free-breathing and MANIV-BH CBCTs. The GTV propagated is compared to the median GTV position of the five trained operators that performed the image-guided RT registration when using (A and B) automatic rigid registration using only translation or (C and D) oART GTV propagation from the planning CT to the CBCT using an image guided deformable registration. The metrics used for the comparison of these two volumes were (A and C) the distance between the CoM (mm) and (B and D) the HD95% (mm). The colours of the points, each representing a CBCT, were determined based on the most frequent CBCT quality score reported by the operators. CoM: Centre of mass, FB: Free breathing, GTV: Gross tumour volume, HD95: 95% Hausdorff distance, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold, oART: Online-adaptive radiotherapy.



#### ***4.2.3.4. Deep-learning auto-segmentation performance***

Once again, CBCT acquisition using MANIV-BH demonstrated superior results compared to free-breathing when evaluating the performance of the deep-learning auto-segmentation algorithm (Excellent or good: 80.0% versus 4.0%,  $p < 0.001$ , Figure 4.2.1C and Figure 4.2.2C). Detailed results for individual OARs are provided in Figure A9.2. The median time required for manual correction of the deep-learning-generated OAR volumes was significantly reduced by 54.2% when using MANIV-BH CBCTs (8.7 minutes [interquartile range: 4.0 – 11.7]) compared to free-breathing CBCTs (19.0 minutes [interquartile range: 15.8 – 22.7],  $p < 0.001$ ).

#### **4.2.4. Discussion**

This study comparing liver SABR CBCTs acquired under free-breathing and MANIV-BH conditions highlights the clinical advantages of MANIV-BH. MANIV-BH significantly enhances CBCT image quality, facilitates image-guided RT, reduces interoperator image-guided RT variability, improves OARs auto-segmentation performance, and decreases the time required for manual edition of these volumes. These improvements are critical for accurate liver SABR delivery, with or without oART. Since MANIV-BH for liver SABR has become routine in our RT department, this study presents the first real-world results of MANIV-BH.

Accurate image-guided RT is essential to prevent geometric misses during liver SABR [517]. Percutaneously implanted radiopaque markers can serve as tumour position surrogates and registration targets between planning CT

and CBCT [180, 518]. They are more accurate than anatomical surrogates such as the diaphragmatic dome [181, 483]. However, their use can be limited by patient factors, tumour location, and risks such as bleeding, pain, infection, or tumour seeding [182, 486]. Also, they may cause artefacts on follow-up imaging, making their interpretation difficult. MRI-guided RT offers another alternative, enabling direct tumour visualisation, smaller PTV margins, and beam interruption when the tumour moves outside the PTV [183-185, 519]. However, MRI-guided RT is resource-intensive and not widely available [187]. While still uncommon, MANIV-BH is more accessible and less expensive than MR-linacs, making it a promising option.

Beyond the subjective benefits of improved image quality and easier registration, this study shows that MANIV-BH reduces registration uncertainties among trained operators. These findings align with previous lung SABR studies, where respiratory motion-mitigating techniques improved CBCT image quality and reduced interoperator image-guided RT variability [520-522]. Minimising image-guided RT variability remains crucial for tumour control and toxicity reduction. Strategies include optimising CBCT reconstruction, using modern imaging systems, and advanced image-guided RT training [522]. In this study, image-guided RT variability was significantly higher among non-trained operators, particularly for MANIV-BH CBCTs (Figure A9.1), underscoring the need for adequate training in such high-precision treatments, as demonstrated in other RT contexts [523, 524].

During CBCT-guided oART, manually editing auto-segmented OAR volumes is among the most time-consuming steps. Previous *in silico* CBCT-guided

oART studies reported average editing times of 24.0 minutes for locally advanced pancreatic cancer (within 36.3-minute sessions) and 11.4 minutes for abdominal metastases (22.6-minute sessions) [313, 314]. In clinical treatments of gastric lymphoma, Zhong et al. reported an average session duration of 25.0 +/- 6.8 minutes, with OAR and target volume refinement taking up most of the time [525]. Gardner et al. highlighted that improved CBCT quality reduces interoperator variability in OAR delineation, potentially lowering correction time during oART, though this remains to be confirmed [526]. Our study demonstrates that higher CBCT quality not only improves OAR auto-segmentation but may halve correction time (8.7 versus 19.0 minutes). This reduction offers several benefits: shorter oART sessions, reduced staffing needs, fewer intrafraction changes, and potential improvements in outcomes like lower toxicity risk [308, 527, 528]. Additionally, shorter sessions may enhance patient comfort and offer economic advantages.

Unlike passive respiratory motion management techniques, MANIV enables stable apneas [176-179, 529], reducing breathing-related tumour motion uncertainties and allowing PTV margins reduction [178, 530]. Other non-invasive ventilation modes, like shallow-controlled ventilation (high rate with low amplitude), also reduce PTV margins [177, 531]. However, only MANIV-BH eliminates respiratory motion artefacts during CBCT acquisition, which is not possible with continuous-breathing techniques. Deep, stable inspiration apneas also provide dosimetric advantages by reducing OARs dose, and improve interfraction positional reproducibility compared to voluntary breath-hold [179]. Practical limitations include longer RT sessions

(25 – 40 minutes), though comparable or shorter than gating or tracking techniques [532]. It also requires expertise and well-trained team [178, 179]. We are actively developing solutions to support wider adoption of this technique.

This study has some limitations, mainly due to its *in silico* design, particularly for image-guided RT registration and manual correction of auto-segmented OAR volumes. Ideally, these tasks should have been performed using the Ethos® treatment platform to accurately reflect our clinical practice. However, the Ethos® emulator - which allows for the virtual delivery of both non-adaptive and oART sessions - was not available at the time of the study. Consequently, image-guided RT registration and OAR auto-segmentation tasks were performed using treatment planning systems different from those used clinically, and without time constraints inherent to real-life RT sessions with a patient on the couch. Therefore, absolute image-guided RT variability and OAR correction time values should be interpreted cautiously, while the relative benefits of MANIV-BH over free-breathing CBCTs remain evident. Lastly, for automatic GTV propagation, the free-breathing group compared to MANIV-BH differed from the free-breathing group used in other analyses.

#### **4.2.5. Conclusion**

CBCTs acquired using MANIV-BH demonstrated superior quality compared to those obtained under free-breathing conditions during liver SABR. High-quality CBCTs offer key advantages in conventional RT by improving image-

guided RT precision, reducing interoperator variability for trained staff (radiation oncologists and radiotherapy technologists), and making the process more comfortable. MANVI-BH also shows promise for CBCT-guided oART, as it improves the performance of deep-learning-based automatic delineation of OARs and reduces the time required for manual corrections of these volumes. Beyond the benefits of CBCT quality improvement, MANIV-BH also offers the advantage of inducing stable apneas during irradiation, which reduces tumour motion uncertainties and allows for PTV margin reductions. These advantages should further encourage teams to work on the current limitations of the MANIV-BH technique, enhance its dissemination and continue improving it, particularly in terms of dedicated time.

### **4.3. Radiotherapy in MSS colorectal cancer: immune impact of the dose and the anatomical site**

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*This work is not published (See Appendix 10 for supplementary data).*

## **Highlights**

- Preoperative immunomodulatory RT is safe and feasible in CRC
- RT increase proportion of circulating terminally differentiated effector memory RA<sup>+</sup> (TEMRA) CD4<sup>+</sup> cells
- Immune infiltration is similar in 3 x 8 Gy compared to non-irradiated lesions
- RT activates cell cycle and immune pathways in tumours
- Subgroup analyses limited by low numbers of lung, primary, Low-RT, and out-of-field lesions

## **Abstract**

### *Background*

The prognosis of metastatic microsatellite-stable colorectal cancer (MSS CRC) remains poor. Immunotherapy provides only modest benefit, and clinical trials combining radiotherapy (RT) with immune checkpoint inhibitors have yielded disappointing results. However, these trials were conducted without robust biological evidence in humans regarding the immune effects of RT according to dose and anatomical site. This study investigated the immune impact of preoperative RT in MSS CRC, in addition to its feasibility and safety.

### *Materials and methods*

Patients with primary MSS CRC or metastases scheduled for surgery were prospectively enrolled. RT to the lesion scheduled for resection was delivered 4-8 days before surgery. A moderate RT dose (3 x 8 Gy, Mod-RT) was compared with matched non-irradiated lesions from a historical cohort (No-RT). Additional lesions, when present, received low-dose RT (3 x 1 – 2 Gy, Low-RT) or no RT (Out-RT). Tumours were also stratified by anatomical site (primary, liver, non-liver metastases). Peripheral blood was collected before and after RT for immune cell phenotyping, plasma proteomics, and TCR sequencing. Tumour samples underwent multiplex immunofluorescence and RNA sequencing to characterise the immune microenvironment.



## *Results*

Seventeen patients (11 liver, 2 lung, 3 primary, 1 combined primary + liver) were included, allowing collection of 18 Mod-RT lesions, 18 matched No-RT lesions, 2 Low-RT, and 5 Out-RT lesions. Preoperative RT was safe and feasible. Comparison of pre- and post-RT blood samples revealed no major changes in circulating immune cell subsets, T-cell activation/exhaustion status, or plasma immune protein profiles, with the exception of an increase in CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells and a slight increase of the clonality of the TCR repertoire. T cell subpopulations (CD8<sup>+</sup>, CD45RO<sup>+</sup>, PD-1<sup>+</sup>) and immunoscore within tumours did not differ between Mod-RT and No-RT lesions. Other subgroup analyses (lung, primary, Low-RT, Out-RT) were underpowered.

## *Conclusion*

Preoperative RT for immunomodulation purposes in MSS CRC is safe and feasible but induces limited systemic and local immune modulation. Larger studies are required to clarify dose- and site-specific effects.

#### **4.3.1. Introduction**

Colorectal cancer (CRC) is the fourth most common cancer worldwide and the second leading cause of cancer-related death, accounting for approximately 905,000 deaths annually [1]. Despite advances in oligometastatic resection, biomolecular classification, and systemic therapies, the prognosis of metastatic CRC remains poor, with a 5-year overall survival of 14% [125, 126]. This is of particular concern given that 22% of patients present with metastatic disease at diagnosis, and 20 – 50% of initially non-metastatic cases will develop metastases over time [125].

An important exception for this poor prognosis is observed in mismatch repair-deficient/high microsatellite instability (dMMR/MSI-H) tumours, which benefit significantly from immune checkpoint inhibitors compared to conventional chemotherapy and targeted therapies [147, 148]. However, these tumours represent only 15% of all CRCs and 3 – 5% of metastatic cases [44]. In contrast, the majority of CRCs are microsatellite stable (MSS) and poorly responsive to immunotherapy, which is not recommended in this setting [39, 153, 155, 157]. Compared to dMMR/MSI-H tumours, MSS tumours exhibit a low tumour mutational burden, reduced neoantigen load, and limited infiltration of antitumoral immune cells such as CD4<sup>+</sup> and CD8<sup>+</sup> T cells [46, 82].

Improving the immunogenicity of MSS tumors is a major research focus to enhance immunotherapy efficacy [533]. Preclinical studies have shown that radiotherapy (RT) can modulate the tumour immune microenvironment and prime T cells, potentially improving responses to immune checkpoint

inhibitors [234, 533]. However, translating these findings into clinical results is difficult. Clinical trials of RT-immunotherapy combination in metastatic MSS CRC have so far yielded disappointing results [534-536].

To bridge this gap, critical RT parameters, such as dose, volume, and timing relative to immunotherapy, must be optimised in order to induce the strongest and the most beneficial immune reaction [234, 537]. The irradiation site also appears to play a key role. In CRC, the liver is the most frequent site of metastasis [27]. However liver metastases are associated with systemic immunosuppression that impairs immunotherapy efficacy [76, 154]. RT to liver metastases has been shown in preclinical models to reverse this immunosuppression and restore immunotherapy sensitivity [538]. Finally, identifying novel immune targets remains essential to develop immunotherapy agents that synergise effectively with RT. Since RT parameters and immune targets can vary from mice to human, translational in vivo studies remain crucial to understand RT-induced immune modulation in human MSS CRC [533].

The objective of this study is to evaluate the modulation of the tumour immune microenvironment induced by RT, according to both RT dose and the site of irradiation, in patients with MSS CRC. The selection of the radiotherapy dose, irradiated volumes, and timing between radiotherapy and surgery was carefully considered and is detailed in Section 1.5.4 of this manuscript. As this protocol involves RT delivered shortly before scheduled surgery, which is uncommon, the feasibility and safety of this approach are also assessed.

## **4.3.2. Material and Methods**

### ***4.3.2.1. Study design***

This ongoing prospective, translational, multicentric window-of-opportunity study has enrolled patients between September 2023 and July 2025. It aims to collect tumour and blood samples to assess the impact of different RT doses and irradiated anatomical sites on the local and systemic immune environment in patients with MSS CRC. Ethical approval was obtained from the ethics committees of Cliniques Universitaires Saint-Luc and CHU UCL Namur (National registration number: B4032023000027). All patients provided written informed consent, and all procedures were conducted in accordance with the Declaration of Helsinki. An overview of the study design is presented in Figure 4.3.1.

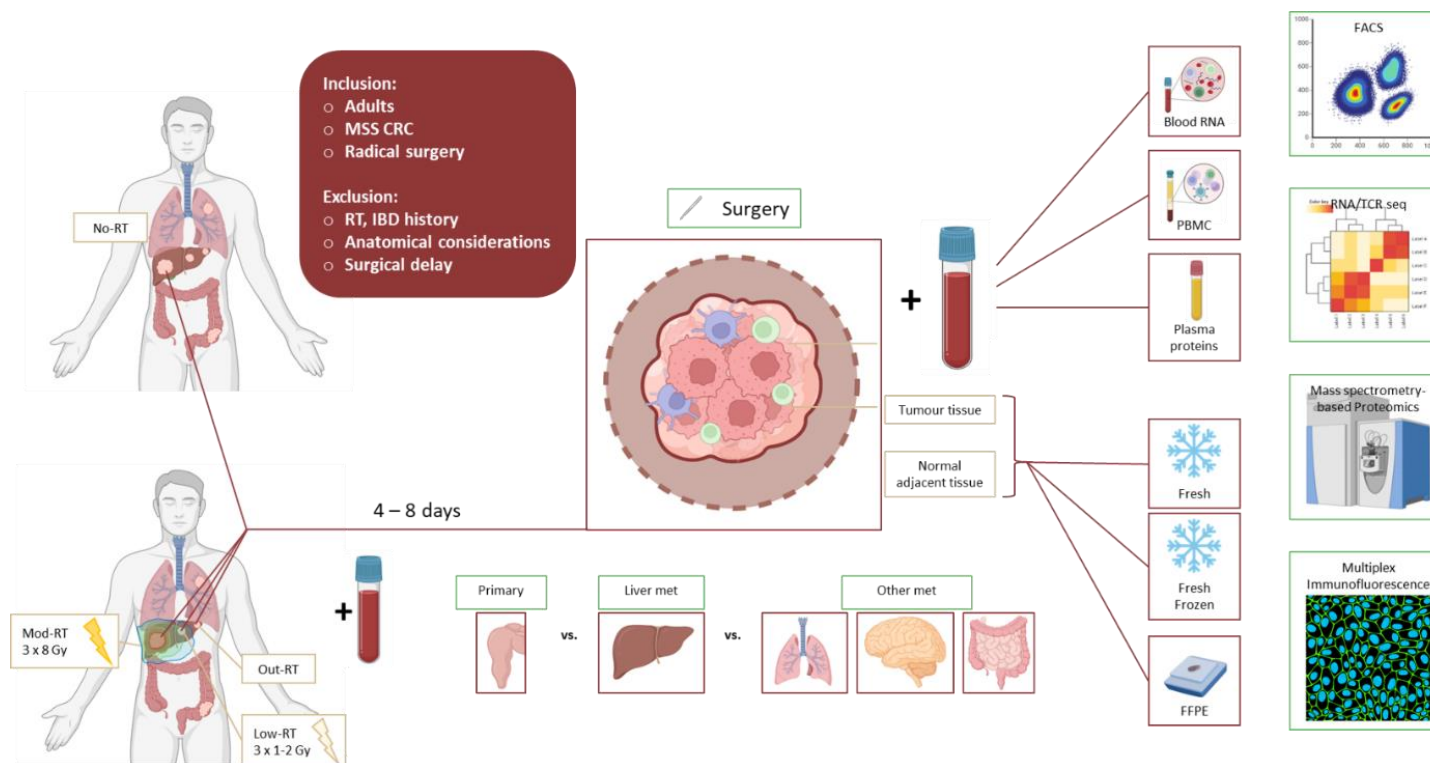


Figure 4.3.1: Overview of the study procedures. CRC: Colorectal cancer, FACS: Fluorescence-activated cell sorting, FFPE: Formalin-fixed paraffin-embedded, IBD: Inflammatory bowel disease, MSS: Microsatellite stable, PBMC: Peripheral blood mononuclear cell, RNA: Ribonucleic acid, RT: Radiotherapy, TCR: T cell receptor.

#### *4.3.2.2. Patients*

Patient selection was conducted in agreement with the surgeon, radiologist, radiation oncologist, and referring medical oncologist during the colorectal multidisciplinary tumour board, where the indication for radical surgery was confirmed. Inclusion criteria were: (1) non-pregnant adults (>18 years), (2) biopsy-confirmed MSS CRC, (3) scheduled for radical surgery, and (4) presence of one or more CRC metastases. Since March 2024, the protocol has been amended to include non-metastatic patients scheduled for primary tumour resection. Exclusion criteria included prior RT to the surgical area or a history of inflammatory bowel disease (for primary tumour cases). Anatomical contraindications were lesions abutting critical vascular structures or small tumours (<10 mm in long axis on medical imaging), which could compromise tissue sampling or RT accuracy. As a window-of-opportunity study, patients with surgery scheduled within 10 days of the tumour board discussion were not considered eligible to avoid delaying standard oncologic care.

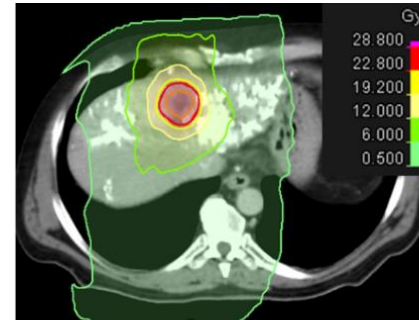
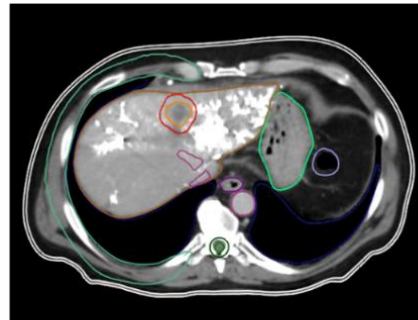
#### *4.3.2.3. Procedures*

For experimental RT, planning CT was acquired with the patient in a supine position. Iodine contrast was used only for patients with liver metastases RT. For liver and lung metastases RT, a 4D-CT with audio-coaching was performed to account for respiratory motion, and a mid-position approach was used to define PTV margins [173, 174]. In cases of colon tumours, motion mitigation strategies included a 2-hour fasting period, oral butylhyoscine (20mg), and online-adaptive RT [280]. RT was planned using

the RayStation system (version 12A, RaySearch Laboratories, Stockholm, Sweden) and delivered using intensity-modulated techniques on three linear accelerators (Halcyon® and Ethos®, Varian, a Siemens Healthineers company, Palo Alto, USA; Elekta Infinity, Elekta AB, Stockholm, Sweden). Daily cone-beam CT was acquired for image-guided RT. RT was completed 4 to 8 days before surgery to allow observation of immune modifications prior the peak of RT-induced fibrosis, typically occurring around two weeks post-RT.

A dose of 24 Gy in three daily fractions was delivered to one CRC lesion (primary or metastatic), defining the “Mod-RT” group. In patients undergoing multiple resections, additional lesions received lower doses (3 x 1-2 Gy, “Low-RT”) or no RT (3 x 0 Gy, “Out-RT”), depending on proximity to the high-dose target (illustrated in Figure 4.3.2). Gross tumour volume (GTVs) was delineated using multimodal imaging from the preoperative work-up; no clinical target volume (CTV) margins were applied. Planning objectives are detailed in Table A10.1. Strict dose constraints were applied to highly vascularized organs (e.g., blood vessels, spleen, heart) and tumour-draining lymph nodes (e.g., hepatic or pulmonary hilum) to preserve tumour-specific immune responses.

**Mod-RT**



**Out-RT**

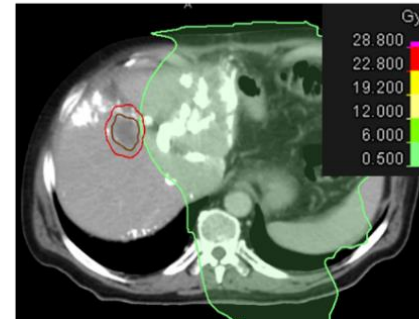
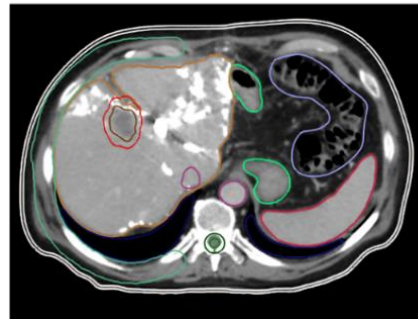


Figure 4.3.2: Illustration of a patient included in the study. He was irradiated on a liver lesion in segments II-IV at a dose of  $3 \times 8$  Gy (Mod-RT). A second lesion located in segments IV-VIII was used as an avoidance volume during the RT planning, ensuring a mean dose lower than 0.5 Gy (Out-RT). The patient underwent a left hepatectomy 5 days after RT completion. RT: Radiotherapy.



Surgical resections were performed by a specialised surgical team including thoracic (n=1), liver (n=3), and colorectal (n=2) surgeons. Immediately after tumour resection, specimens were promptly transferred to the pathology laboratory to enable rapid processing of fresh and fresh-frozen tissue, completed within 30 minutes (Figure 4.3.2). Blood samples were collected prior to RT initiation (Pre-RT) and at the start of the surgical procedure (Post-RT).

Tumour samples from never-irradiated CRC patients enrolled in an ongoing prospective study at our institution (National registration number: B4032020000038) served as a fourth comparison group (“No-RT”). These patients were optimally matched to those in the current study based on age, sex, tumour location (right colon, left colon, or rectum), mutational status (*KRAS* and *BRAF*), and preoperative treatment (Table A10.2).

#### **4.3.2.4. Feasibility and safety**

Feasibility and safety outcomes were assessed across the RT, surgical, and follow-up phases. For RT, acute adverse events occurring between RT initiation and surgery were recorded using CTCAE v5.0 [539]. Target volume coverage and dose to organs at risk were also analysed. Surgical outcomes included intraoperative complications, procedure duration, blood loss, resection margin status, length of hospital stay, and postoperative complications. Surgeons involved in the study completed a feasibility perception survey to provide feedback.

No additional study-specific visits were scheduled after surgery; follow-up data were obtained through routine oncological care. Postoperative outcomes included 30-day morbidity, 90-day mortality, and recurrence rates. Quantitative data were reported as medians with interquartile ranges.

#### *4.3.2.5. Fluorescence-activated cell sorting (FACS)*

Peripheral blood was collected in Mononuclear Cell Preparation Tubes, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Solution at 1600 x g for 28 minutes at room temperature. Plasma was removed and stored at -20°C, and PBMCs were washed twice by centrifugation in PBS at 1300 rpm for 10 minutes at 4°C. Cells were then resuspended in IMDM medium supplemented with 10 – 20% heat-inactivated human serum and 1% penicillin/streptomycin, filtered through a 0.2 µm membrane. Then, they were stored at -80°C in 1 ml aliquots containing at least 10<sup>6</sup> viable cells, following the addition of 10% DMSO.

After thawing in a 37 °C water bath, PBMCs were gradually transferred dropwise into PBS and washed twice by centrifugation at 500 x g for 10 minutes at 4°C. Cells were then stained with fluorescent antibodies for 10 minutes at 4°C and resuspended in 300 µl PBS for FACS analysis. Peripheral blood mononuclear cell phenotyping was performed using the NovoCyte Quanteon System. Two panels were used: one for characterising the major immune cell populations, and a second for assessing T cell activation and exhaustion (Figure 4.3.3). A brief summary of the role of each cellular

subpopulation analysed in the first panel in tumour immunogenicity is provided in Section 1.2.3.1.

Data were analysed with FlowJo™ (version 10.10.0) and the T-REX (Tracking Responders Expanding) algorithm [540]. Within-patient differences (Post-RT - Pre-RT) were checked for normality with the Shapiro-Wilk test. Normally distributed differences were analysed with a two-tailed paired Student's t-test; non-normal data were analysed with the Wilcoxon signed-rank test. A multiplicity correction was performed using the Benjamini-Hochberg method. P-values < 0.05 were considered significant. Immune-cell proportions were visualised with the ComplexHeatmap package (version 2.22.0) in R (version 4.4.2). Unsupervised hierarchically clustered was performed using the Ward's method.

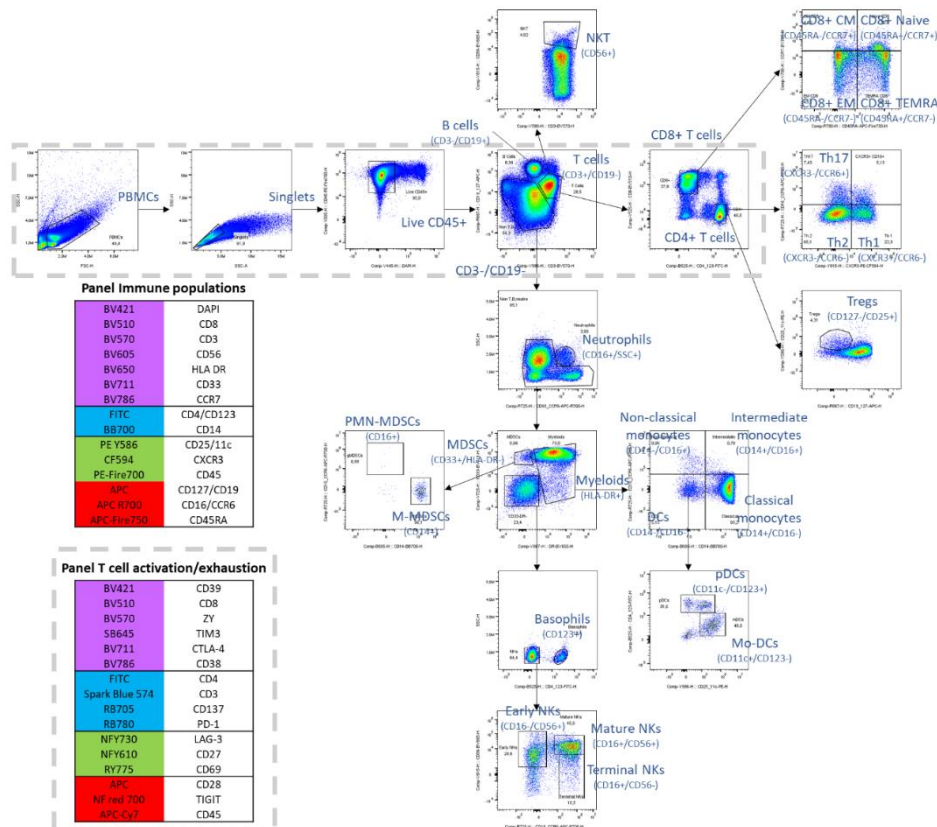


Figure 4.3.3: Gating strategies for immune blood cell analyses performed using fluorescence-activated cell sorting. The gating strategy for the T cell activation/exhaustion panel was identical to that of the immune population panel up to the definition of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. APC: Allophycocyanine, BB: Brilliant Blue, BV: Brilliant Violet, CF: Cross-Fluor, CM: Central memory, (Mo-/p-)DCs: (myeloid/plasmacytoid) Dendritic cells, EM: Effector Memory, FITC: Fluoresceine isothiocyanate, (PMN-/M-)MDSCs : (polymorphonuclear/monocytic) Myeloid-Derived Suppressor Cells, LAG-3 : Lymphocyte Activation Gene-3, NF red: Near-infrared, NFY: Near-Far-Yellow, NKs: Natural killer cells, NKT: Natural killer T cells, PBMCs: Peripheral blood mononuclear cells, PD-1: Programmed cell-death protein 1, PE: Phycoerythrin, RB: Red-brilliant, RY: Red-yellow, SB: Starbright, TEMRA : Terminally-differentiated effector memory RA<sup>+</sup>, Th: T helper, TIGIT: T-cell

*immunoreceptor with Ig & ITIM domains, TIM3: T-cell immunoglobulin and mucin-domain 3, Tregs: Regulatory T cells, ZY: Zombie Yellow.*

#### **4.3.2.6. TCR sequencing**

During pre-RT and post-RT blood collection, samples were drawn into PAXgene Blood RNA Tubes and immediately frozen. All available samples from the study cohort were processed simultaneously using the PAXgene Blood RNA Kit for RNA extraction. After thawing, samples were incubated at room temperature for 2 hours to ensure complete lysis of blood cells. Cellular debris was removed by two centrifugation steps at 5,000 x g for 10 minutes. Protein digestion was performed using proteinase K (10 minutes at 55 °C), followed by DNA removal using DNase I (15 minutes at 20 – 30 °C). RNA was then isolated in 80 µL of RNase-free water through successive buffer addition and centrifugation steps, according to the manufacturer's protocol. RNA concentration was quantified using a NanoDrop spectrophotometer.

Following RNA extraction, samples were shipped to an external provider (Laboratory of Translational Oncology, Grand Hôpital de Charleroi / Institut de Pathologie et de Génétique, Gosselies) for targeted T-cell receptor (TCR) repertoire sequencing and bioinformatic processing. Library preparation was performed using unique molecular identifiers and internal synthetic TRBV spike-in controls, followed by next-generation sequencing. Sequencing reads underwent standard quality-control filtering, including removal of low-quality and contaminant reads. Unique molecular identifier–collapsed

reads were generated for downstream analyses, and samples with insufficient read depth were excluded.

Repertoire diversity metrics were computed by the provider after read-depth normalization, including richness (number of distinct clonotypes), Shannon diversity (an entropy-based index reflecting clonotype richness and evenness), and Simpson diversity (a dominance-weighted index emphasizing highly expanded clonotypes). Paired pre-RT versus post-RT comparisons were performed to assess differential clonotype abundance and clonal expansion or contraction. Differential abundance testing was conducted on clonotype counts between matched samples using thresholds of  $|\log_2 \text{fold change}| \geq 2$  and  $p < 0.05$ , with multiple-testing correction applied using the Benjamini–Hochberg method. Statistical analyses and data visualization were performed using the *ggpubr* package (version 0.6.0) in R (version 4.4.2).

#### 4.3.2.7. *Plasma proteome*

A total of 100  $\mu\text{L}$  of frozen plasma, isolated from peripheral blood collected for FACS analysis, was used for mass spectrometry-based proteomic profiling. Plasma samples were first depleted of high-abundance proteins using the High Select Top14 Abundant Protein Depletion Resin to enhance the detection of low-abundance proteins. Protein preparation involved reduction, alkylation, trichloroacetic acid precipitation, and washing with cold acetone, followed by digestion with sequencing-grade trypsin. Peptides were fractionated into seven fractions and subsequently pooled into three

fractions using the Pierce High pH Reversed-Phase Peptide Fractionation Kit to reduce sample complexity. Each pooled fraction was analysed on an Orbitrap Exploris 240 mass spectrometer. Data-independent acquisition was performed, and DIA-NN (version 1.8.1) was used to distinguish true signals from analytical noise and to generate an *in silico* predicted spectral library from the UniProtKB human reference proteome [541, 542]. Bioinformatic analyses were carried out in R (version 4.4.2). The three fractions per sample were pooled, normalized, log-transformed, and peptides were aggregated into proteins using the QFeatures package (version 1.16.0) [543]. Contaminants were removed using the cRAP database (camptoR package, version 0.0.0.9000) and a personalised list. Differential expression analysis was performed with the limma package (version 3.62.2) using a linear model that included timepoint relative to RT (Pre-RT versus Post-RT) and irradiated site as experimental factors, while accounting for paired samples from the same patients. Enrichment analysis were performed using the msigdbR package (version 24.1.0) to investigate cancer hallmarks and reactome pathways. Further details on the plasma proteomics analysis are provided in [544].

#### 4.3.2.8. *Multiplex immunofluorescence*

Sections (5 µm) of formalin-fixed paraffin-embedded tumour tissue were mounted on SuperFrost Plus slides and dried for at least 24 h. Slides were deparaffinized with toluene and isopropanol, followed by inactivation of endogenous peroxidase activity using 3% hydrogen peroxide in isopropanol

for 20 min. Sequential staining of each target was then performed, including: antigen retrieval, blocking of non-specific binding, primary antibody incubation, secondary antibody incubation, and tyramide signal amplification. Between each substep, slides were washed three times in TBS/0.1% Tween, except between blocking and primary antibody application. A panel including anti-CD3, anti-CD8, anti-CD45RO, anti-PD-1, and anti-PD-L1 previously developed and optimised was used (Table A10.3 and Figure 4.3.7A) [545].

Antigen retrieval was performed in either a basic solution (25 ml 10 mM EDTA, pH 9, supplemented with 225 ml distilled H<sub>2</sub>O and 675 µl Triton) or an acidic solution (25 ml 10 mM citrate, pH 5.7, with 225 ml distilled H<sub>2</sub>O and 675 µl Triton), depending on the antibody (Table A10.2). Slides were immersed and heated by microwave: until boiling (around 4.5 minutes at 900 W), followed by 15 minutes at 90 W, and re-boiling (around 1.5 minutes at 900 W). Blocking of non-specific binding was performed with 5% bovine serum albumin (BSA) in TBS/0.1% Tween for 30 minutes at room temperature. Primary antibodies (diluted in TBS/0.1% Tween/1% BSA) were incubated for 60 minutes, followed by incubation with secondary anti-mouse or anti-rabbit antibodies for 40 minutes (Table A10.3). Tyramide amplification was performed by applying a fluorochrome-tyramide reagent in borate buffer (0.1 M borate, pH 7.8, 3 M NaCl) containing 0.001% H<sub>2</sub>O<sub>2</sub> for 10 minutes.

After the final staining cycle, antigen retrieval with acidic buffer was repeated, followed by nuclear counterstaining with Hoechst 33342 (20 mM,



diluted 1:1000 in H<sub>2</sub>O) for 5 minutes. Slides were then washed and manually mounted with fluorescence mounting medium and covered by borosilicate glass.

Slides were scanned on a Zeiss Axioscan.Z1 slide scanner at 20x magnification using custom filter sets. Image analysis was performed in QuPath (version 0.6.0). Tumour regions were manually delineated and expanded by 500 µm to include the invasive margin. Automated cell and nucleus segmentation was performed with InstantSeg (version 0.1.1), and segmentation quality was verified visually on at least 50 cells per slide [546]. Cells were automatically classified using intensity thresholding for each marker.

Further methodological details on multiplex immunofluorescence are available in [545].

For the immunoscore computation, the densities of CD3<sup>+</sup> and CD8<sup>+</sup> cells were quantified for each sample in the tumour core and invasive margin (Table A10.3 and Figure 4.3.7A). For each region and marker, immune cell density was computed as the number of positive cells divided by the annotated area (cells/mm<sup>2</sup>). Densities were then converted into percentiles relative to the distribution observed across the whole cohort within the same region and marker. The mean of the four resulting percentiles was defined as the immunoscore, which was subsequently categorized into “Low” ( $\leq 25\%$ ), Intermediate (25 - 70%), or High ( $> 70\%$ ) [83].

#### 4.3.2.9. RNA-sequencing

Five formalin-fixed paraffin-embedded sections of 5 µm per tumour samples were acquired for RNA extraction using the RNeasy FFPE Kit. After deparaffinisation, tissue digestion was performed using proteinase K (15 minutes at 56°C followed by 15 minutes at 80°C and then cooled on ice for 3 minutes). Elimination of DNA was performed using DNase I (15 minutes at room temperature). RNA was then isolated in 40 µl of RNase-free water using different cycles of buffer addition and centrifugation according to the vendor procedure. RNA concentration was quantified using a Nanodrop spectrophotometer. Sample with a DV200 > 50% were considered for library preparation. The integrity of the extracted RNA was measured on a 2200 TapeStation system. The library preparation was performed using a Watchmaker RNA Polaris Depletion - rRNA/Globin HMR (FFPE) kit. Sample with a concentration > 10 mM at the quality control were considered for sequencing with a NovaSeq X Plus sequencer. Integrity analysis, library preparation, and RNA sequencing steps were subcontracted by Macrogen®.

The quality control of the sequencing (fastq files) was performed bioinformatically using FastQC (version 0.12.1). The alignment of the fastq file was performed using HISAT2 (version 2.2.1) using the human reference genome GRCh38 [547]. The resulting BAM files were processed using featureCounts (version 2.1.1) to generate the count matrix [548].

The differential gene expression analysis was performed using R (version 4.4.2) with package DESeq2 (version 1.46.0), considering adjusted p-value < 0.05 and Log2 Fold change of +/- 1.5 as statistically significant [549]. Gene

enrichment analysis was performed using the cancer hallmark list from the msigdb package (version 24.1.0) and by using publications of gene signatures related to tumour immune microenvironment composition, response to immunotherapy, and prognosis in CRC [550-556]. Deconvolution to evaluate immune populations in the tumour samples was performed using CIBERSORTx [557, 558].

Resource references are available in Table A10.4.

### **4.3.3. Results**

#### **4.3.3.1. Patients**

A total of 114 patients were screened for eligibility. Of the initial 22 patients included in the study, 4 did not undergo RT and were subsequently excluded. This resulted in 18 patients who completed preoperative RT. Among these, 10 underwent liver metastasis resection, 3 underwent lung metastasis resection, 3 underwent primary tumour resection, and 2 underwent combined liver metastasis and primary tumour resection (Figure 4.3.4 and Table 4.3.1).

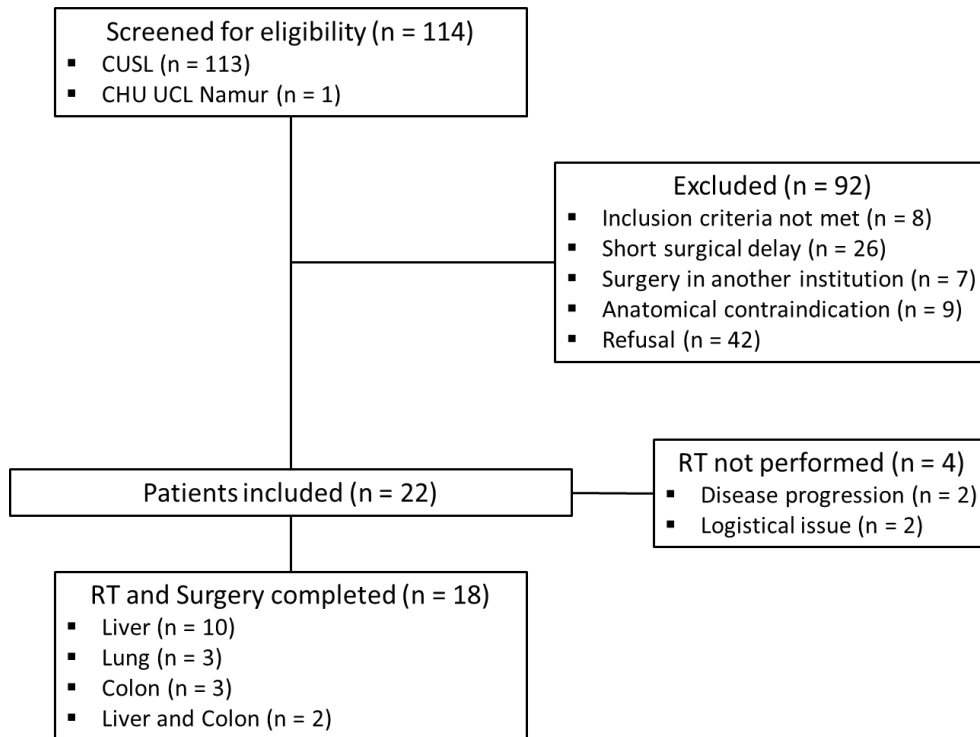


Figure 4.3.4: Flow chart of the study. CUSL: Cliniques Universitaires Saint-Luc, RT: Radiotherapy.

| Patients | Gender | Age | PS | BMI  | Comorbidities                                                                                                                                                         | Irradiated lesion                                                           | Radiotherapy group          |
|----------|--------|-----|----|------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------|
| P01      | M      | 72  | 0  | 24.4 | Atrial fibrillation<br>CIDP<br>Glaucoma<br>MGUS                                                                                                                       | Liver – Segment VII<br>Liver – Segment IV<br>Liver – Segment IV             | High-RT<br>Low-RT<br>Out-RT |
| P02      | M      | 60  | 0  | 31.3 | -                                                                                                                                                                     | Liver – Segment II-IV<br>Liver – Segment IV-VIII                            | High-RT<br>Out-RT           |
| P03      | F      | 73  | 1  | 29.8 | Arterial hypertension<br>Atrial fibrillation<br>Breast cancer history<br>Chronic renal failure<br>Hashimoto thyroiditis<br>Long-QT syndrome<br>Thromboembolic disease | Liver – Segment III<br>Liver – Segment I-II-III<br>Liver – Segment I-II-III | High-RT<br>Low-RT<br>Out-RT |
| P04      | F      | 62  | 1  | 34.2 | Factor V Leiden                                                                                                                                                       | Liver – Segment V-VI<br>Liver – Segment VII                                 | High-RT<br>Out-RT           |
| P05      | M      | 68  | 0  | 28.1 | Atrial fibrillation<br>Epilepsy                                                                                                                                       | Liver – Segment V                                                           | High-RT                     |
| P06      | M      | 75  | 0  | 23.9 | Arterial hypertension<br>Chronic renal failure                                                                                                                        | Lung – Superior right lobe                                                  | High-RT                     |

|      |   |    |   |      |                                                                 |                                                        |                    |
|------|---|----|---|------|-----------------------------------------------------------------|--------------------------------------------------------|--------------------|
|      |   |    |   |      | Diabetes mellitus<br>Ischemic cardiopathy<br>Pulmonary embolism |                                                        |                    |
| P07  | F | 66 | 0 | 16.7 | MGUS                                                            | Liver – Segment VI                                     | High-RT            |
| P08  | F | 58 | 0 | 21.3 | Hypothyroidy                                                    | Liver – Segment V-VI-VII-VIII                          | High-RT            |
| P09  | M | 69 | 0 | 27.3 | Chronic renal failure<br>Ischemic cardiopathy                   | Lung – Superior left lobe<br>Lung – Inferior left lobe | High-RT<br>Out-RT  |
| P10* | F | 36 | 0 | 24.7 | -                                                               | Liver – Segment VI                                     | High-RT            |
| P11  | M | 60 | 0 | 19.9 | Arterial hypertension                                           | Liver – Segment III-IV                                 | High-RT            |
| P12  | M | 74 | 0 | 34.7 | Arterial hypertension<br>COPD                                   | Left colon                                             | High-RT            |
| P13  | M | 78 | 0 | 28.7 | Aortic prothesis<br>Chronic pancreatitis<br>Diabetes mellitus   | Sigmoid colon                                          | High-RT            |
| P14  | F | 75 | 2 | 18.1 | Arterial hypertension<br>Fibromyalgia<br>Hypothyroidy           | Left Colon<br>Liver – Segment VI                       | High-RT<br>High-RT |
| P15  | F | 81 | 1 | 22.7 | Transient ischemic<br>attack                                    | Left Colon                                             | High-RT            |
| P16  | M | 67 | 0 | 23.8 | Arterial hypertension                                           | Liver – Segment VIII                                   | High-RT            |

|       |   |    |   |      |                              |                           |         |
|-------|---|----|---|------|------------------------------|---------------------------|---------|
|       |   |    |   |      | Benign prostatic hyperplasia |                           |         |
| P17** | F | 57 | 0 | 23.4 | Ulcerative colitis           | Lung – Inferior left lobe | High-RT |
|       |   |    |   |      | Arterial hypertension        |                           |         |
| P18   | M | 63 | 0 | 32.8 | Chronic renal failure        | Liver – Segment III       | High-RT |
|       |   |    |   |      | Diabetes mellitus            |                           |         |

*Table 4.3.1: Characteristics of the included patients. \* This patient received concomitant short-course pelvic radiotherapy (5 x 5 Gy) and was therefore included in the “Liver and colon” group for blood analyses. However, only the liver sample was retained for tumour tissue analyses. \*\*Pathological evaluation of the lung lesion revealed a benign process. Consequently, this patient was included only in the feasibility and safety analyses, but excluded from the translational analyses on blood and tumour. BMI: Body mass index, CIDP: Chronic inflammatory demyelinating polyneuropathy, COPD: chronic obstructive pulmonary disease, F: Female, M: Male, MGUS: Monoclonal gammopathy of undetermined significance, PS: Performance status.*

#### **4.3.3.2. Feasibility and safety results**

*(Detailed results on the feasibility and safety of preoperative RT are provided in Appendix 10.1.)*

For RT, among the 100 evaluated target volume coverage objectives, 84.0% were successfully met (Table A10.5). All organs-at-risk constraints were respected, except for one patient whose liver metastasis was adjacent to the stomach and duodenum. In this case, dose constraints could not be met; however, prophylactic treatment with oral ondansetron (8 mg) and pantoprazole (40 mg) prevented RT-related toxicity. Four patients experienced Grade 1 fatigue, and one had Grade 2 vomiting.

RT did not delay surgery in any patient. Complete resection was achieved in 88.5% (23/26) of lesions. One patient (P17), initially diagnosed with a lung metastasis, was excluded from the translational analysis after pathology confirmed a benign lesion. No intraoperative complications occurred, except for one case of aeric leakage. None of the postoperative complications were attributed to preoperative RT (Table A10.6). While all surveyed surgeons initially expressed concerns regarding RT-related surgical complexity, these were alleviated after performing at least one procedure (Figure A10.1).

Median follow-up was 9.0 months (interquartile range: 5.0 – 12.8). The 30-day morbidity rate was 11.1%, including one case of persistent postoperative pain and one of severe malnutrition. No deaths occurred within 90 days. Four patients experienced local recurrence, and six had distant progression. At the latest follow-up, all patients were alive.

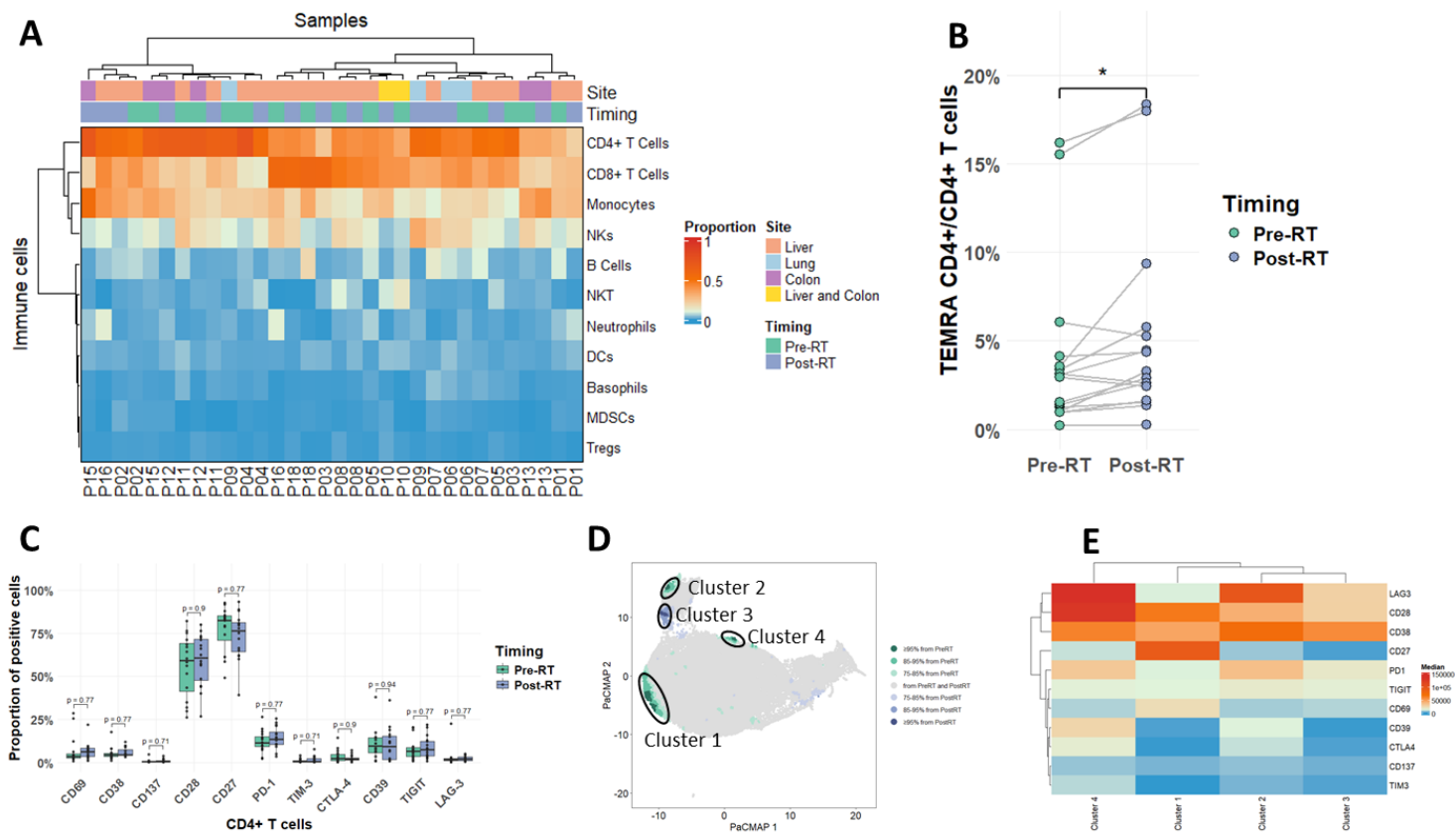


#### 4.3.3.3. *FACS: Radiotherapy increases circulating terminally differentiated effector memory RA<sup>+</sup> (TEMRA) CD4<sup>+</sup> cells*

Blood acquired before RT initiation was compared with blood obtained after RT, at the time of surgery, to evaluate immune cell populations by FACS. Sample from one patient (P14, irradiated on both the primary tumour and liver metastasis) could not be analysed due to a technical issue during preprocessing and/or freezing. The first panel identified major circulating immune cell populations (CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B cells, Tregs, neutrophils, basophils, monocytes, Myeloid-derived suppressor cells, dendritic cells, natural killer cells, and natural killer T cells; the role of these cells in antitumour immunity is described in Section 1.2.3.1.). No significant differences were observed between Pre-RT and Post-RT samples for any of these populations ( $p > 0.05$ , Figure 4.3.5A). Within these general populations, subpopulations were further characterized (Figure 4.3.3). Among them, only the proportion of terminally differentiated effector memory RA<sup>+</sup> CD4<sup>+</sup> T cells within total CD4<sup>+</sup> T cells was significantly higher after RT ( $p = 0.049$ , Figure 4.3.5B, Appendix 10.2). When restricting the analysis to patients irradiated on liver metastases only ( $n = 10$ ), no significant differences were observed ( $p > 0.05$ , Figure A10.2A).

The second panel evaluated activation (CD69, CD38, CD137), exhaustion (PD-1, TIM-3, CTLA-4, CD39, TIGIT, LAG-3), and maturation (CD27, CD28) markers on CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 4.3.3). No significant differences in the expression of individual markers were observed between Pre-RT and Post-RT samples for either CD4<sup>+</sup> ( $p > 0.05$ , Figure 4.3.5C) or CD8<sup>+</sup> T cells ( $p >$

0.05, Figure 4.3.5F). To further explore marker expression patterns, dimensionality reduction with PaCMAP followed by unsupervised k-nearest neighbour clustering (T-REX algorithm) was applied to CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Pre-RT versus Post-RT). For CD4<sup>+</sup> T cells, four clusters were identified: clusters 1, 2, and 4 were enriched in Pre-RT samples, while cluster 3 was enriched in Post-RT samples (Figure 4.3.5D). Cluster 1 showed high CD27 and CD28 expression and low expression of other markers, consistent with a naïve phenotype. Clusters 2 and 4 exhibited reduced CD27 expression and increased inhibitory markers (PD-1, CTLA-4, CD39, LAG-3), suggestive of an exhausted phenotype. In contrast, cluster 3 was characterised by reduced naïve markers (CD27, CD28) but also reduced inhibitory markers (PD-1, TIM-3, CTLA-4, CD39, LAG-3, Figure 4.3.5E), which is compatible with terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells. For CD8<sup>+</sup> T cells, two clusters were identified, both predominantly represented in Pre-RT blood (Figure 4.3.5G). Cluster 1 showed high expression of inhibitory markers (PD-1, CTLA-4, CD39, LAG-3) together with CD137, but reduced expression of other activation markers (CD69, CD38) and maturation markers (CD27, CD28) ( $p < 0.001$ , Dunn's test). Cluster 2 was characterized by high CD27 expression and low expression of most other markers except CD137, CD69, and CTLA-4, suggestive of a naïve phenotype ( $p < 0.001$ , Dunn's test, Figure 4.3.5H). Slight differences exist for patients irradiated on liver metastasis only (Appendix 10.2, Figure A10.2B-G).



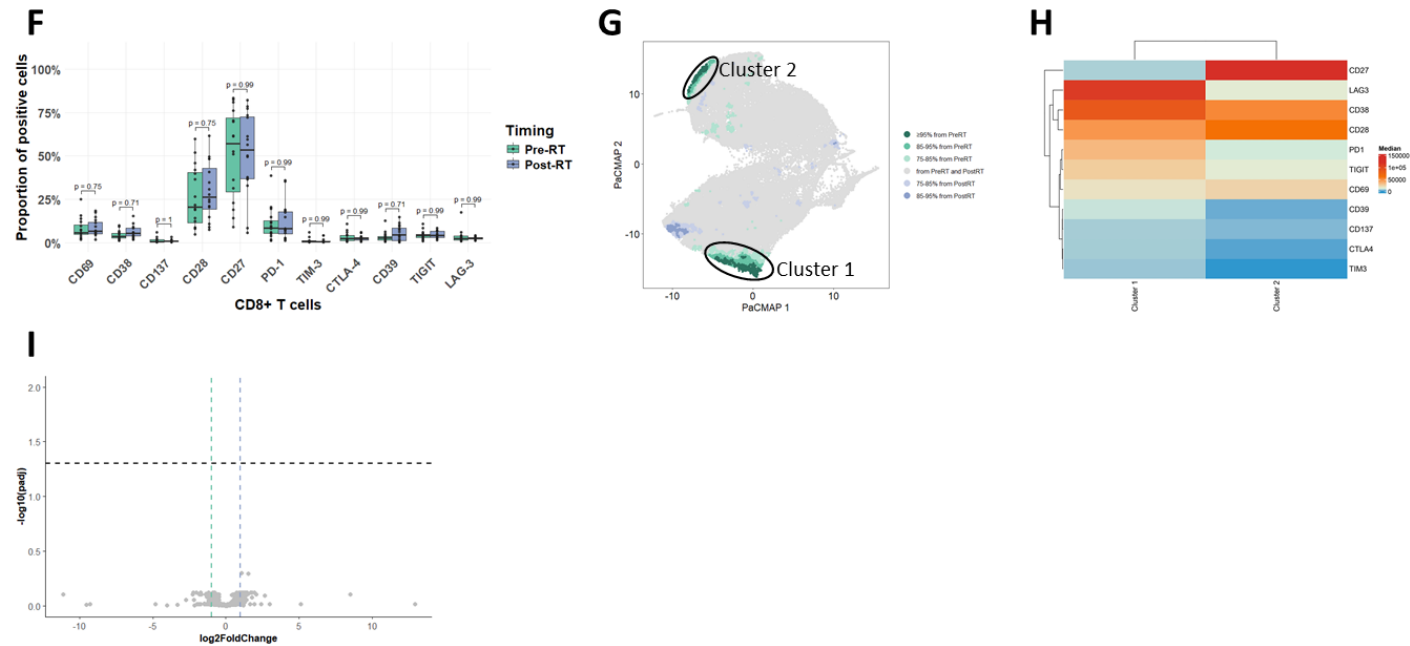


Figure 4.3.5: (A) Proportions of circulating immune cell populations according to RT timing (Pre-RT versus Post-RT) and irradiation site. (B) After irradiation, the proportion of terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells within the CD4<sup>+</sup> T-cell population significantly increased. (C) Single expression of activation/exhaustion markers in CD4<sup>+</sup> T cells comparing Pre-RT and Post-RT blood. (D) Dimensionality reduction with unsupervised clustering of CD4<sup>+</sup> T cells based on activation/exhaustion marker expression identified four clusters. Clusters 1, 2, and 4 were enriched in Pre-RT blood, while cluster 3 was enriched in Post-RT blood. (E) Expression of

activation/exhaustion markers within each CD4<sup>+</sup> T-cell cluster. (F) Single expression of activation/exhaustion markers in CD8<sup>+</sup> T cells comparing Pre-RT and Post-RT blood. (G) Dimensionality reduction with unsupervised clustering of CD8<sup>+</sup> T cells based on activation/exhaustion marker expression identified two clusters, both comprising cells from Pre-RT blood. (H) Expression of activation/exhaustion markers within each CD8<sup>+</sup> T-cell cluster. (I) Differential analysis of plasmatic proteins showed no significant differences between Pre-RT and Post-RT blood. \*  $p$ -value < 0.05. RT: Radiotherapy.

#### *4.3.3.4. TCR sequencing: a slight increase in clonality following radiotherapy*

RNA from paired pre-RT and post-RT blood samples was analysed for T-cell receptor (TCR) sequencing in 15 patients. For three patients, one of the two samples yielded an insufficient number of reads for reliable analysis; these patients (P07, P11, and P15) were therefore excluded from the paired comparative analysis. Diversity analyses showed a decrease in TCR repertoire richness (not significant,  $p > 0.05$ ; Figure 4.3.6A), Shannon diversity (significant,  $p = 0.007$ , Figure 4.3.6B), and Simpson diversity (not significant,  $p > 0.05$ , Figure 4.3.6C) in post-RT samples compared with pre-RT samples. In addition, a significantly higher number of overrepresented clonotypes was observed in post-RT blood relative to pre-RT blood (paired Wilcoxon signed-rank test,  $p = 0.047$ ; Figure 4.3.6D).

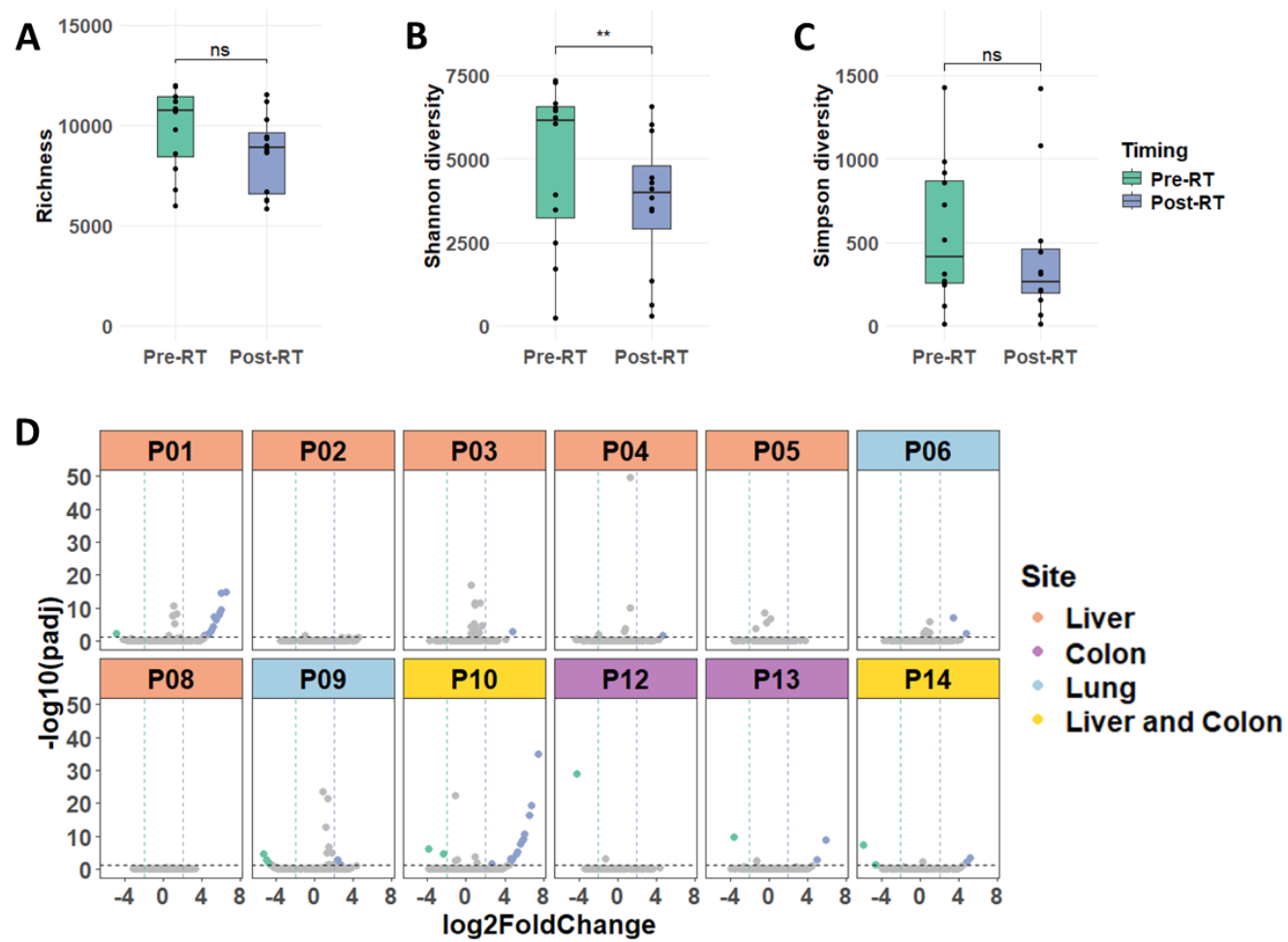


*Figure 4.3.6: TCR sequencing results. Diversity metrics, including (A) richness, (B) Shannon diversity, and (C) Simpson diversity, decreased after irradiation, with statistical significance observed only for Shannon diversity. (D) A higher number of clonotypes was overrepresented in post-RT blood samples compared with pre-RT samples. Taken together, these findings suggest that RT induces a modest clonal expansion of the T-cell receptor repertoire. RT: Radiotherapy, TCR: T-cell receptor. ns: not significant, \*\*  $p < 0.01$ .*



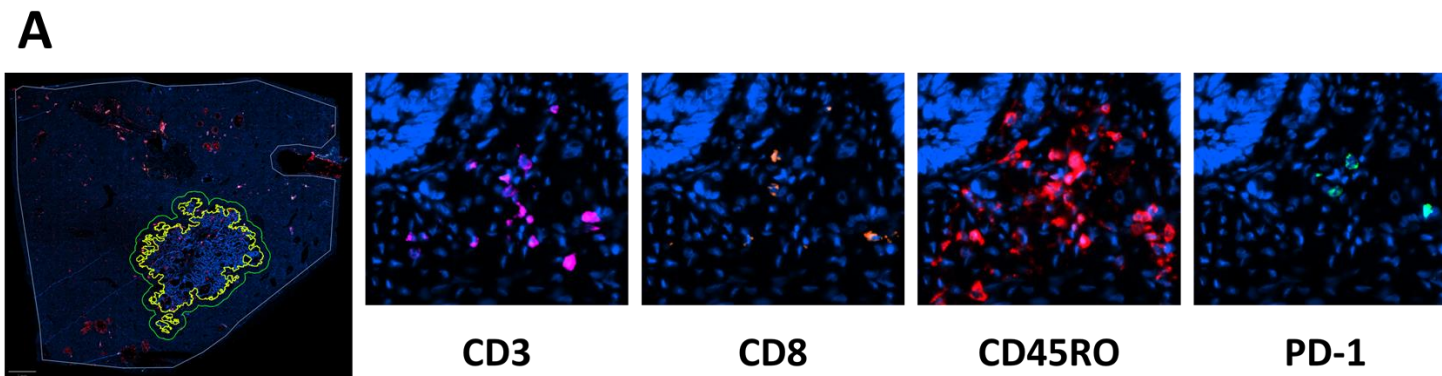
#### *4.3.3.5. Plasma proteomics: Only p53 pathway-associated proteins are overexpressed in post-RT plasma*

Data were available for all patients except one (P18). Analysis of individual protein concentrations revealed no significant differences between Pre-RT and Post-RT blood samples ( $p > 0.05$ , Figure 4.3.5I). Only four cytokines (TGfree-breathing1, KITLG, interleukin-18, and CSF1) were detected in both pre-RT and post-RT samples, and none showed significant differential expression ( $p > 0.05$ ). However, proteins associated with the p53 pathway were significantly more abundant in Post-RT blood compared with Pre-RT samples ( $p = 0.009$ , details in Appendix 10.3). No reactome pathways were differentially expressed between Pre-RT and Post-RT blood ( $p > 0.05$ ). Similar results were obtained when restricting the analysis to patients irradiated on liver metastases only, although in this subgroup the overexpression of the p53 pathway in post-RT blood did not reach statistical significance ( $p = 0.055$ , Figure A10.2H).

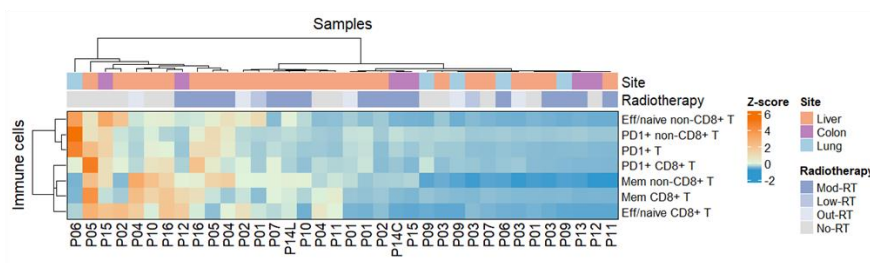
#### *4.3.3.6. Multiplex immunofluorescence: Lack of evident influence of irradiation and anatomical site on tumour microenvironment immune cell density*

Multiplex immunofluorescence was performed on tumour samples from the Mod-RT group ( $n = 18$ ) and on paired tumours from the No-RT group (3 matched from the same patients and 15 from different matched patients, Table A10.2). In addition, Low-RT tumour samples were available for 2

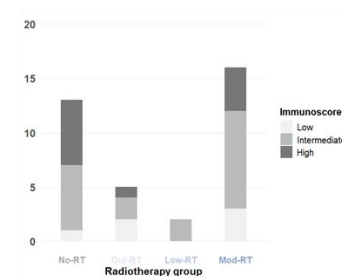
patients, and Out-RT tumour samples were available for 5 patients. Although panel 1 included PD-L1, its quantification was not interpretable due to very scarce staining (Figure 4.3.7A). Of the 43 multiplex slides, 36 (83.7%) were interpretable, while the remaining 7 showed too few tumour cells and/or extensive necrosis. When considering all tumour samples, no clear patterns emerged across RT groups or irradiation sites in the composition of T-cell subsets (CD3<sup>+</sup>), whether cytotoxic (CD8<sup>+</sup>), memory (CD45RO<sup>+</sup>), or PD-1<sup>+</sup> (Figure 4.3.7B). Comparisons between Mod-RT and No-RT lesions did not reveal significant differences in tumour immune microenvironment composition, either across all anatomical sites ( $p > 0.05$ , Student's t-test, Figure 4.3.7C and Figure 4.3.7E) or when restricted to liver metastases ( $p > 0.05$ , Student t-test, Figure 4.3.7G and Figure 4.3.7H). Although the number of Low-RT and Out-RT lesions was small, analyses restricted to these patients did not suggest major differences in tumour immune microenvironment composition compared with Mod-RT and No-RT lesions (Figure 4.3.7J and Figure 4.3.7K). For each sample, the immunoscore was computed as the mean percentile of CD3<sup>+</sup> and CD8<sup>+</sup> cell densities in both the tumour core and the invasive margin, calculated relative to all included samples, and categorized as "Low" ( $\leq 25\%$ ), "Intermediate" (25-70%), and "High" ( $>70\%$ , Figure 4.3.7C). No significant differences in immunoscore class distribution were observed between Mod-RT and No-RT samples, either across all lesions ( $p > 0.05$ , Stuart-Maxwell test, Figure 4.3.7F) or when restricted to liver metastases ( $p > 0.05$ , Stuart-Maxwell test, Figure 4.3.7I).



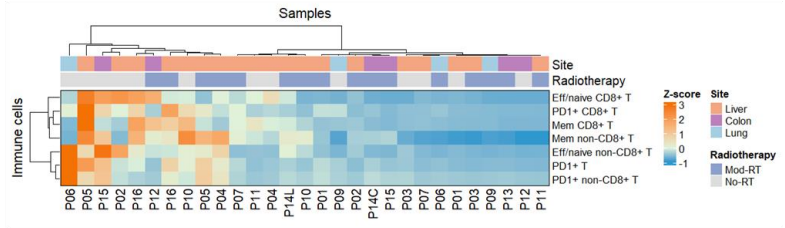
**B All sites - All RT groups**



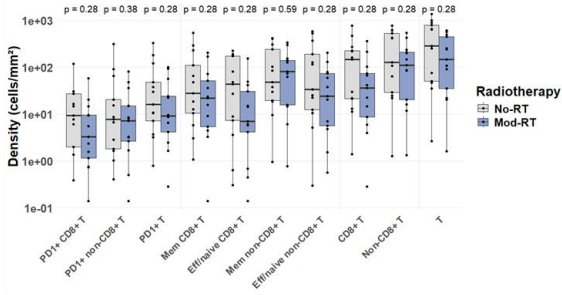
**C**



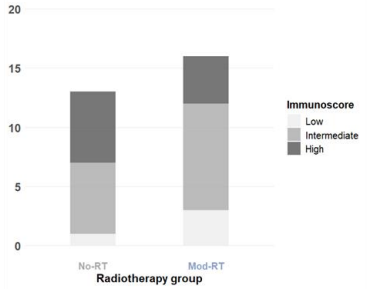
**D All sites – Mod-RT versus No-RT**



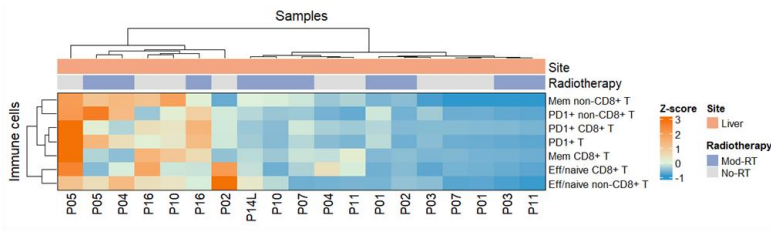
**E**



**F**



**G Liver metastases – Mod-RT versus No-RT**



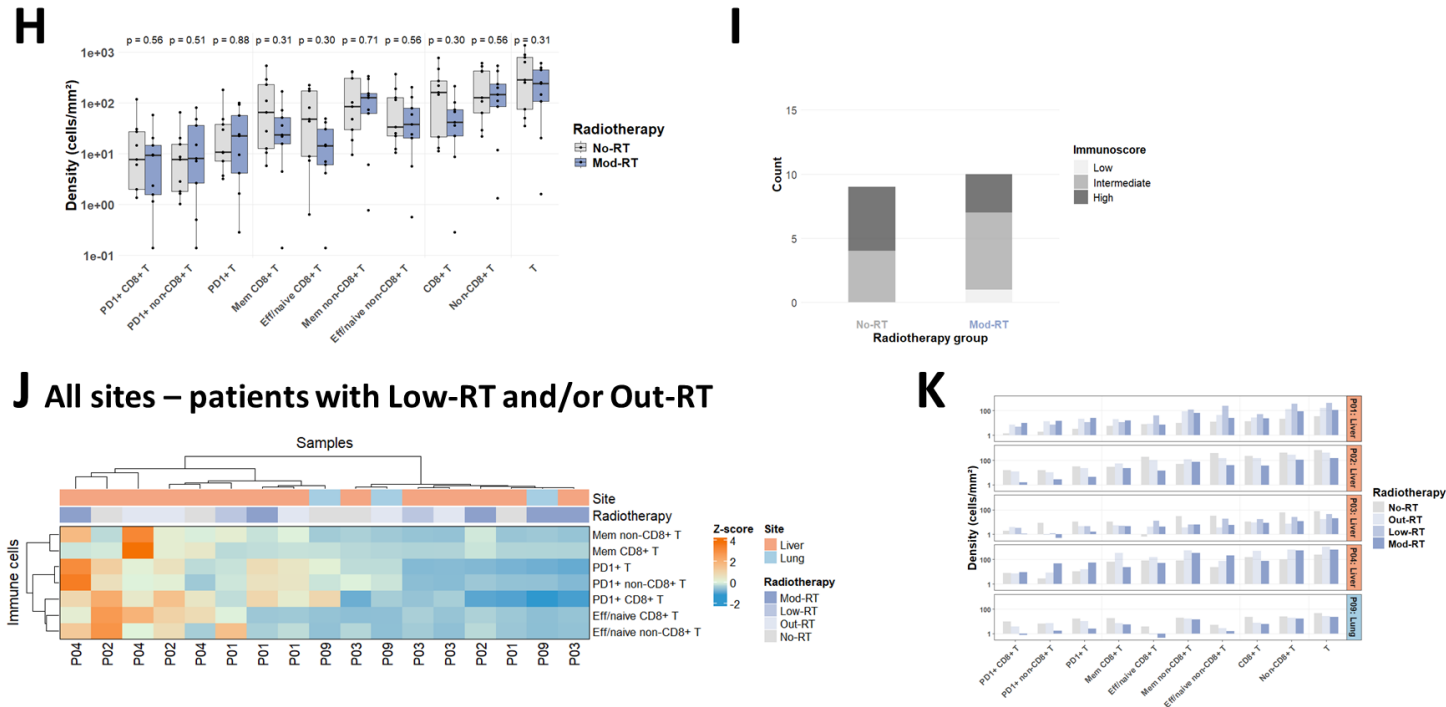


Figure 4.3.7: Multiplex immunofluorescence was performed on tumour samples. (A) The panel was designed to profile T-cell subtypes, enabling the characterization of immune populations within the tumour (yellow line) and in the adjacent invasive margin defined as 500  $\mu$ m (green line). (B) All samples considered together, the tumour immune microenvironment composition seems to be not impacted by the irradiation status nor by the anatomical

site. (C) Distribution of immunoscore categories (“Low”:  $\leq 25\%$ , “Intermediate”: 25 – 70%, “High”:  $>70\%$ ) across all included samples. (D and E) No significant differences in tumour immune microenvironment composition and (F) immunoscore distribution were observed between Mod-RT (3 x 8 Gy) and No-RT lesions, irrespective of irradiation site (non-irradiated patients;  $p > 0.05$ , Student’s t-test). (G–I) The same conclusions applied when analyses were restricted to liver lesions only ( $p > 0.05$ , Student’s t-test). (J and K) Two Low-RT (3 x 1-2 Gy) and five Out-RT (not irradiated in an irradiated patient) lesions were available for comparison with “Mod-RT” and “No-RT” matched-lesion. No clear trends about tumour immune microenvironment composition can be visualised). Eff: Effector, Mem: Memory, RT: Radiotherapy.

#### *4.3.3.7. RNA sequencing: Moderate irradiation limits cell division and stimulate immune pathways*

RNA sequencing was performed on 10 samples (4 Mod-RT, 4 Non-RT, and 2 Out-RT from patients P02, P04, P05, and P07). RNA integrity was suboptimal in 2/10 samples; however, given DV200 values close to 50% (>45%), library preparation was pursued. Library quality control failed in 3/10 samples due to insufficient RNA concentration. Since two samples were near the required threshold (>9 nM versus 10 nM) and the third displayed a satisfactory electrophoretic profile, all libraries were sequenced. The mean sequencing yield was 16.2 +/- 1.1 billion bases.

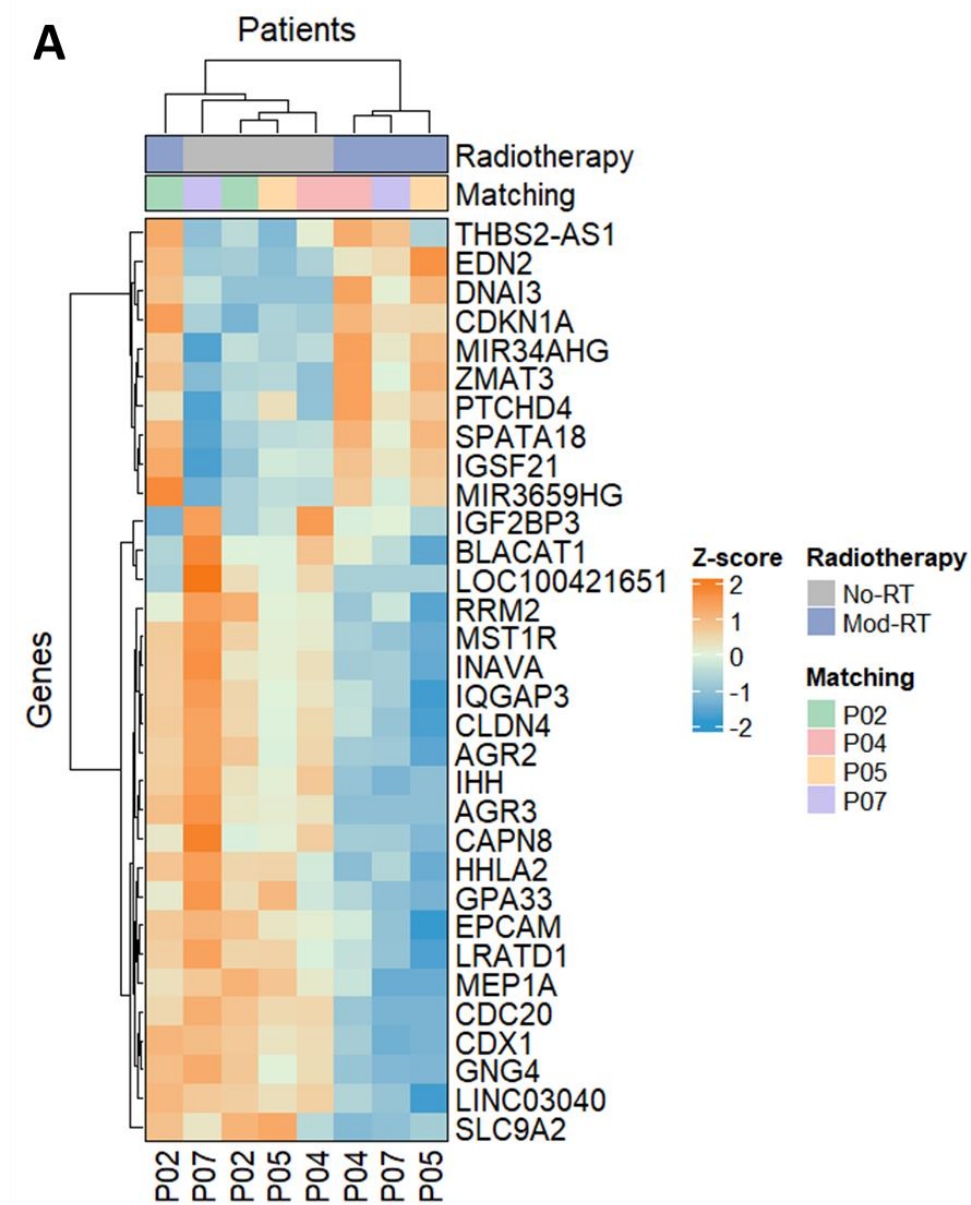
Differential expression analysis between Mod-RT and Non-RT samples identified 38 genes (Figure 4.3.8A-B and Table A10.8). Among them, 11 were implicated in cell cycle regulation, with some genes associated with tumour-promoting proliferation in Non-RT samples (*AGR2*, *AGR3*, *BLACAT1*, *CDC20*, *IGF2BP3*, *RRM2*), whereas others restricted proliferation through RT-induced apoptosis in Mod-RT tumours (*CDKN1A*, *GNG4*, *PTCHD4*, *SPATA18*, *ZMAT3*). One Immune-related gene (*IGSF21*) were overexpressed in Mod-RT, while 3 (*HHLA2*, *INAVA*, *MST1R*) were enriched in Non-RT (gene function is described in Table A10.8). Pathway analysis revealed that most differentially represented pathways were associated with immune responses (8/14) and cell cycle regulation (4/14, Figure 4.3.8C). Literature-based gene signature analysis yielded contradictory findings. While signatures suggested reduced immune infiltration in Non-RT tumours (type I, Deng et al. [551]) and increased sensitivity to immune checkpoint

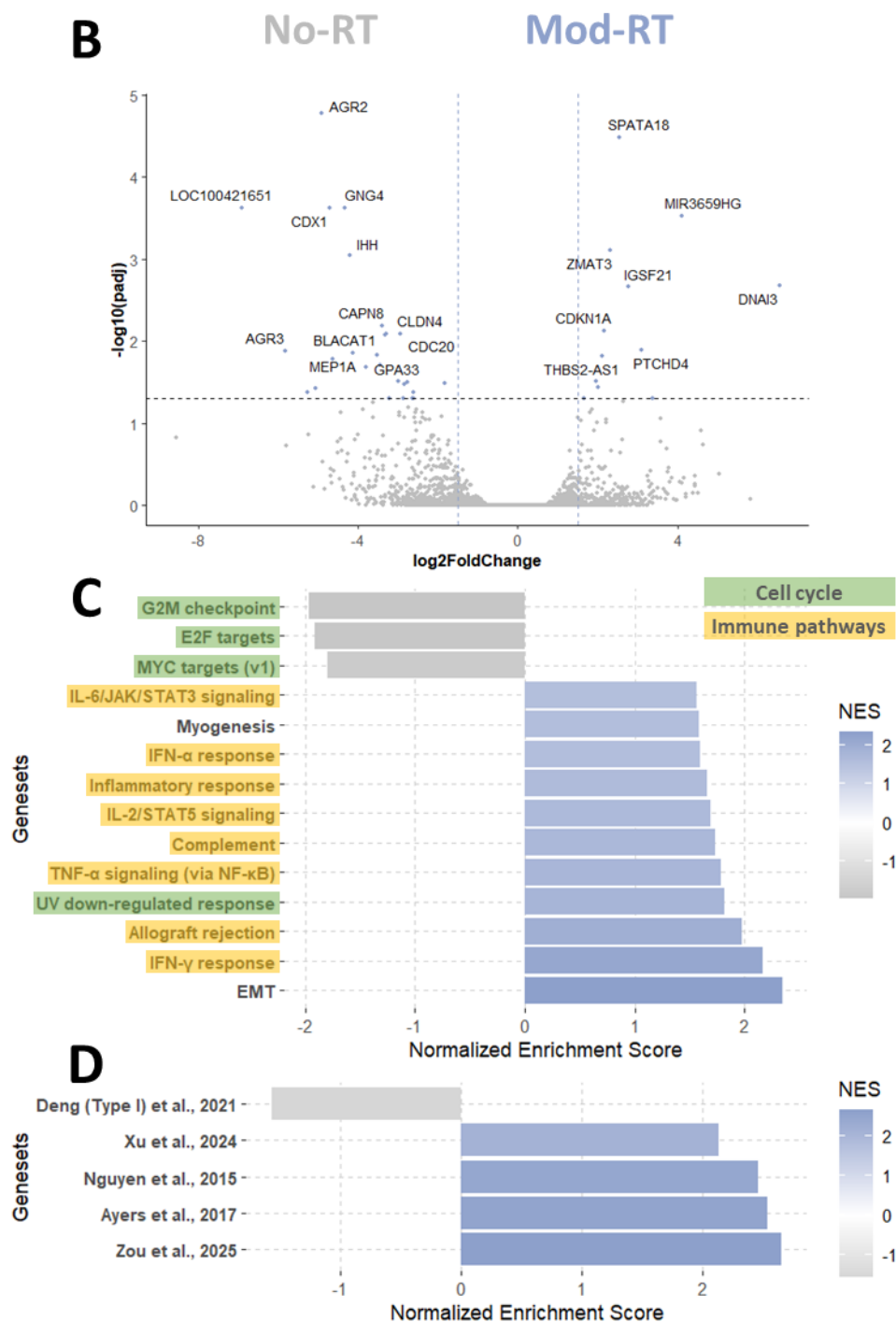
inhibitors in Mod-RT (Ayers et al. [553]), Mod-RT samples more frequently displayed signatures of lack of tertiary lymphoid structures (Xu et al. [552]), poor CRC prognosis (Nguyen et al. [555]), and high cancer-associated fibroblast infiltration (Zou et al. [554], Figure 4.3.8D).

Given the limited number of Out-RT samples (n=2), analyses were exploratory and reported in Appendix 10.3.

Deconvolution analysis did not reveal significant differences in immune cell subtype infiltration across groups (paired Wilcoxon signed-rank test,  $p > 0.05$ , Figure 4.3.8E).







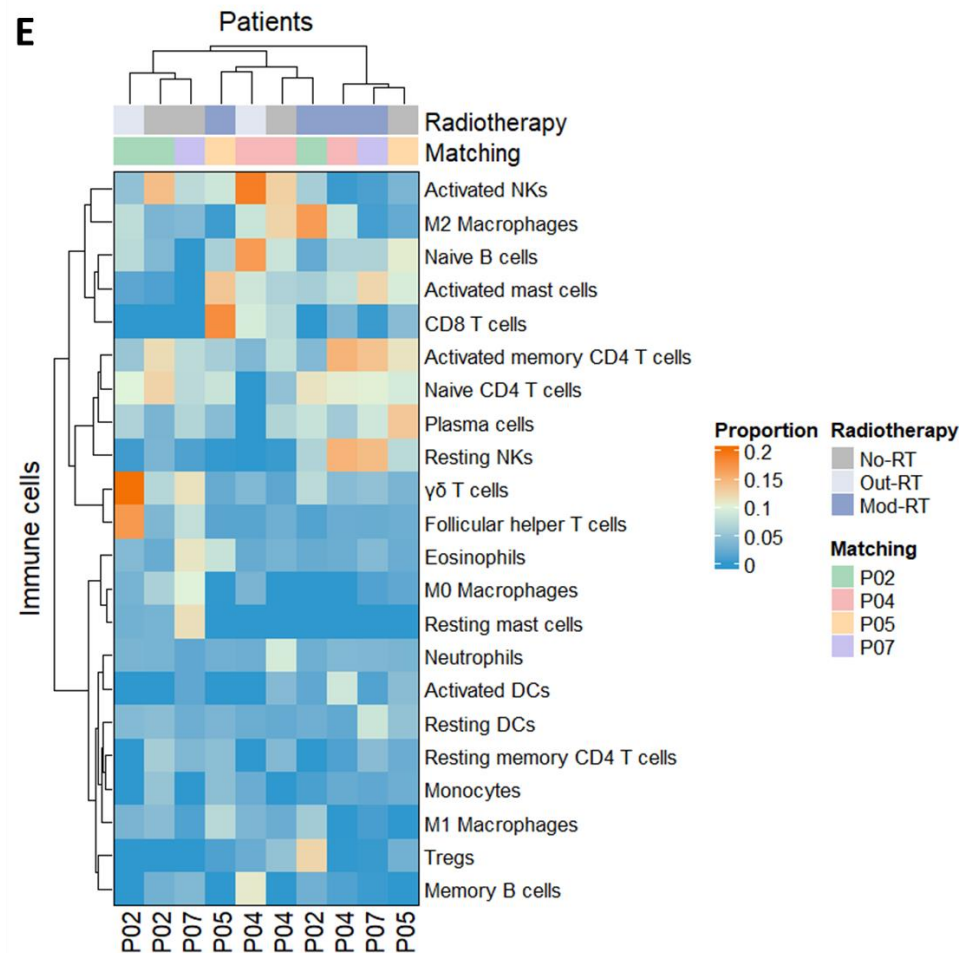


Figure 4.3.8: RNA-sequencing analysis. A total of 38 genes were differentially expressed between Mod-RT and No-RT samples (A and B), 11 of which being involved in cell cycle regulation and 4 in immune reaction. Enrichment analysis was performed using the hallmarks of cancer list (C) and gene signatures retrieved from the literature (D). No significant difference regarding immune cell subtype infiltration assessed using deconvolution analysis by CIBERSORTx (E) was demonstrated between Non-RT, Out-RT and Mod-RT samples (pairwise comparisons, paired Wilcoxon signed-rank test,  $p > 0.05$ ). EMT: Epithelial-mesenchymal transition, IFN: Interferon, RT: Radiotherapy, TNF: Tumour-necrosis factor.

#### **4.3.4. Discussion**

In non-resectable MSS metastatic CRC, current treatment options include chemotherapy and, depending on biomolecular profile, targeted therapies [39]. However, their efficacy remains limited, resulting in poor overall survival outcomes [125]. Hopes for immunotherapy have been disappointed, as multiple clinical trials have failed to show meaningful activity in MSS tumours, in contrast to dMMR/MSI-H CRC [153, 155, 157]. While awaiting a new revolution in cancer treatments, strategies to enhance immunogenicity of MSS tumours leading to the increase of immunotherapy responsiveness are therefore under active investigation. Among these, combining RT with immunotherapy appears promising based on preclinical evidence [204, 233, 234].

In a phase II trial of pretreated MSS metastatic CRC, patients received RT (3 x 8 Gy) on a metastatic lesion concomitantly with nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4), starting on the first day of the second immunotherapy cycle. The per-protocol overall response rate in non-irradiated lesions was 10%, with a median progression-free survival of 2.4 months and a median overall survival of 7.1 months [559]. Similar results were obtained when RT was initiated at the start of the first immunotherapy cycle [560]. Another phase II trial evaluated durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) combined with RT, with non-standardised RT doses across patients. Overall response rate in non-RT lesions was 8.3%, with median progression-free survival of 1.8 months and median overall survival of 11.4 months [535]. Similarly, a phase II trial combining durvalumab and

tremelimumab with liver-directed RT (3 x 10 Gy) in eight metastatic CRC patients (six MSS, one dMMR/MSI-H, one unknown) reported no responses in non-irradiated lesions, with median progression-free survival 2.8 months and median overall survival 16.1 months [536].

These modest clinical results challenge the benefit of combining RT with immune checkpoint inhibitors, despite encouraging preclinical data. The lack of human evidence on the immunologic effects of RT in CRC precludes the identification of the optimal RT regimen (dose, site, timing with immunotherapy) and the most suitable immune targets. This study evaluated the impact of RT dose (3 x 8 Gy versus 3 x 1 – 2 Gy versus unirradiated lesions) and site (liver metastases versus non-liver metastases versus primary tumour) based on extensive biomolecular analyses. It aimed to characterise the tumour immune microenvironment and its modification induced by RT as well as identify immunotherapy targets to be combined with RT. This required operative sampling, raising the question of the safety and feasibility of short-course preoperative RT.

Current knowledge on preoperative RT safety is mainly extrapolated from other settings. In rectal cancer, no significant difference was found between preoperative and postoperative RT in terms of surgical mortality (0.7% versus 1.3%,  $p = 0.41$ ) or postoperative complications (36% versus 34%,  $p = 0.68$ ), including anastomotic leakage, wound healing, or bleeding [100]. In hepatocellular carcinoma, pre-transplant RT did not increase mortality and even reduced 30-day readmission rates compared with non-irradiated patients [561]. However, RT doses, and surgical timing in those contexts

differ from our protocol, limiting comparability. RT triggers early TGF- $\beta$  activation within hours, inducing profibrogenic signals, fibroblast proliferation, extracellular matrix expansion, and collagen deposition. Persistent activation of these mechanisms leads to chronic RT-induced fibrosis [273, 274]. Collagen deposition and profibrotic cytokine expression follow a biphasic pattern: an early peak around two weeks post-RT, a decline, and a chronic phase resurgence. Proinflammatory cytokines such as interleukin-1 follow a similar pattern and contribute to acute RT toxicities [275]. In this protocol, surgery was scheduled 4-8 days post-RT to avoid the early inflammatory peak and reduce RT-related surgical risks. The preoperative RT was deemed feasible, as target volume coverage goals were met in the most of cases, with minimal violations of organ-at-risk constraints, resulting in a low rate of RT-induced adverse events. Moreover, the RT is considered safe based on surgery outcomes both in the immediate post-operative period and in the longer term compared to other reports [562-565].

In our study, blood analyses did not show consistent effects of RT on major circulating immune cell subsets or chemokines. These findings contrast with a study in 46 patients irradiated for liver (n = 29) or lung (n = 17) metastases (CRC in 20/46), which reported increased total T, CD4<sup>+</sup>, and CD8<sup>+</sup> HLA-DR<sup>+</sup> T cells, alongside reduced Tregs and natural killer-T cells. However, in that study, RT doses were higher (3 x 15-20 Gy or 4 x 13.5 Gy), and blood sampling occurred 3-4 weeks post-RT, compared with 4-8 days in our trial [566]. Another study confirmed increases in activated inducible co-stimulatory molecule<sup>+</sup> CD4<sup>+</sup> memory (CD45RO<sup>+</sup>) T cells and decreases in total and

cytotoxic natural killer cells post-irradiation, with no changes in PD-1<sup>+</sup> CD4<sup>+</sup> T cells or Tregs. Cytokine reductions (TNF- $\alpha$ , CXCL10, CCL2, CCL3, CCL5, CCL8) were also noted [567]. By contrast, in our proteomic analysis, only p53 pathway-associated proteins were significantly overexpressed in Post-RT blood, likely reflecting their release from irradiated cells. This pattern is consistent with a direct DNA damage response (RT-induced cell-cycle arrest and apoptosis) rather than a systemic immunomodulatory effect. Nonetheless, this could potentially serve as a biomarker of irradiation exposure [201]. However, in this study, the primary histology varied among patients (no individualised data for CRC), and the RT dose was not standardised [567]. The only circulating cell modification observed after RT in our study was an increased proportion of CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells. This subset can correspond to the sole T cell cluster enriched in Post-RT blood identified by multi-marker analysis of the T cell activation/exhaustion panel. This cluster was characterized by low expression of co-stimulatory molecules (CD27, CD28) and CD39 (effector activity and reduced susceptibility to apoptosis), features compatible with CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells [61, 568]. This population is heterogeneous with some cells displaying direct cytotoxic functions through granzyme B and perforin expression, and also secreting interferon- $\gamma$ , which may potentially confer antitumor properties [61, 569]. Notably, in non-small cell lung cancer, expansion of circulating CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells after RT combined with anti-PD-1 therapy has been associated with improved objective response and overall survival [570]. Our TCR sequencing analyses

demonstrated a slight increase in the clonality of the circulating T-cell repertoire following RT. Similar post-RT increases in T-cell clonality have been reported in other cancer types. In renal cell carcinoma, for example, T-cell clonality was shown to increase transiently, peaking at approximately two weeks after RT and declining thereafter, thereby providing insight into the temporal dynamics of RT-induced immune responses [571]. At the tumour level, preclinical data have demonstrated that RT increases T-cell receptor repertoire clonality, predominantly within the CD8<sup>+</sup> T-cell compartment, with an even more pronounced effect when anti-CTLA-4 immunotherapy is combined with RT [572]. Our analyses were not designed to assess T-cell expansion, a feature associated with effective antitumour immune responses. Assessing markers of cell-cycle activity, such as Ki-67, could help determine whether RT promotes T-cell proliferation [573].

The RT impact on the local tumour immune microenvironment has been extensively documented in preclinical studies [25, 537, 574, 575]. RT induces both antitumour effects (CD8<sup>+</sup> T-cell and natural killer-cell recruitment) and immunosuppressive responses (Treg infiltration). These effects are dose-dependent: low-dose RT (<2 Gy/fraction) limits Treg infiltration and promotes M1 macrophage polarisation, while moderate/high doses (>8 – 12 Gy/fraction) increase Treg recruitment and favour immunosuppressive M2 macrophage phenotypes [25]. In our cohort, no significant differences were found between Mod-RT and No-RT lesions regarding CD8<sup>+</sup>, memory, or PD-1<sup>+</sup> T cell subsets, nor in immunoscore classification, which has prognostic value in metastatic CRC [84]. RNA-sequencing analysis indicates a balance between antitumour immunity and protumour immunosuppression.



Specifically, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  pathways were activated, favouring antitumour responses through recruitment of effector cells, their polarisation toward cytotoxic phenotypes, and the establishment of a chemokine environment supportive of immune activation. These effects were counterbalanced by upregulation of interleukin-2/STAT5 and interleukin-6/JAK/STAT3 signalling, promoting Treg expansion and activity via TGF- $\beta$  [576-580]. Overall, RT did not appear to markedly alter immune cell infiltration in MSS CRC, but rather modulated their functional state. Such nuanced changes, rather than a purely pro- or antitumour shift, highlight opportunities for combination strategies. For instance, preclinical models show that RT combined with STAT3 inhibition enhances systemic antitumour immunity, leading to regression of both irradiated and distant lesions, associated with increased M1/M2 macrophage and CD8<sup>+</sup>/Treg ratios in tumour-draining lymph nodes [581].

Our limited number of Low-RT (n = 2) and Out-RT (n = 5) lesions precluded firm conclusions on these subgroups. Similarly, only 2 lung metastases and 3 primary colorectal tumours were included, limiting assessment by anatomical site. Nonetheless, preclinical evidence indicates that site matters: liver metastases exhibit an immunosuppressive environment (reduced T cell infiltration, increased M2-polarised tumour-associated macrophages), explaining their poor response to immunotherapy [153, 538, 582]. RT to liver metastases may reverse this phenotype by increasing CD8<sup>+</sup> T-cell infiltration, reducing myeloid cells, and promoting an anti-tumour cytokine environment, thereby restoring immunotherapy responsiveness similar to non-liver metastases [538, 582].

The main limitations of this study are the small sample size and heterogeneity. Few lung lesions (n = 2), primary tumours (n = 3), Low-RT lesions (n = 2), and Out-RT lesions (n = 5) were included, making subgroup analyses less robust. Preoperative systemic treatments varied across patients, some having received multiple lines of chemotherapy/targeted therapy, other being free of systemic therapy. The No-RT versus Mod-RT matching was not always optimal with samples for both categories from a same patient being available in a minority of cases. Finally, logistical constraints inherent to routine clinical do not allow fresh tissue processing. While standardisation of the procedures has been developed in our institution, the collection and storage of some samples could have been performed under suboptimal conditions compared to preclinical studies.

#### **4.3.5. Conclusions**

Adding RT to immunotherapy to exploit potential synergistic effects is of particular interest in metastatic MSS CRC, where prognosis remains poor and immune checkpoint inhibitors alone show limited efficacy. Although clinical trials of this combination have yielded disappointing results, they were conducted without strong biological evidence on the immune effects of RT in humans. This study demonstrated the feasibility and safety of delivering RT to primary or metastatic CRC lesions 4 – 8 days before surgery. This design enabled systematic collection of biological samples to assess the impact of RT according to dose (3 x 8 Gy versus 3 x 1 – 2 Gy versus unirradiated lesions) and anatomical site (primary tumour, liver metastases,

or non-liver metastases). Peripheral blood analyses revealed no significant enrichment or depletion of specific immune cell subsets except for terminally differentiated effector memory RA<sup>+</sup> (TEMRA) CD4<sup>+</sup> T cells that are enriched in Post-RT blood, nor changes in the plasma immune protein profile between pre- and post-RT samples. A moderate increase of the clonality of the circulating T-cell repertoire was also demonstrated after RT. Similarly, the immune infiltrate composition did not differ between moderately irradiated lesions (3 x 8 Gy) and lesions from never-irradiated patients. However, transcriptomic analysis suggests that irradiated lesions display a balance between pro- and anti-tumour immune signals, warranting further investigation to identify pharmacological immunotherapy targets for combination with RT. The limited number of lung metastases, primary tumours, unirradiated lesions from irradiated patients, and low-dose RT lesions (3 x 1 – 2 Gy) precluded firm conclusions regarding immune modulation in these subgroups.

## **Chapter 5**

# **Discussion, limitations, and perspectives**

## **5.1. Overview of contributions**

Colorectal cancer (CRC) represents a major public health concern due to its high incidence in Western countries, as well as its rising incidence in developing regions and younger populations [1, 4]. Management and therapeutic objectives differ substantially according to disease stage, particularly the presence or absence of metastases [39, 94].

In locally advanced rectal cancer (LARC), neoadjuvant radiotherapy (RT) followed by surgical resection has significantly improved local disease control [97-99]. The addition of the neoadjuvant chemotherapy in a so-called total neoadjuvant therapy (TNT) has further reduced the risk of distant progression and, in some studies, improved overall survival [102, 104, 115]. Current research efforts focus on preserving quality of life, notably through organ preservation strategies with active surveillance in patients achieving a clinical complete response, thereby potentially omitting surgery [103, 117]. In metastatic CRC, prognosis is largely determined by disease extent. Patients with oligometastatic disease may benefit from curative-intent local treatments, whereas those with disseminated disease require systemic therapy. In both LARC and metastatic CRC contexts, RT is a therapeutic option, though it presents specific challenges that the present work aims to address, at least in part [39, 127, 135, 136].

In LARC, RT dose escalation to the primary tumour may increase the clinical complete response rate, thereby expanding the proportion of patients eligible for non-operative management [162, 163]. Delivering this boost remains technically challenging due to rectal anatomy (proximity to critical

pelvic organs-at-risk, OARs) and physiology (rectum motion) [292, 295]. To address these challenges, cone-beam computed tomography (CBCT)-guided online-adaptive RT (oART) has been evaluated, enabling dose escalation to the primary tumour while limiting pelvic toxicities and accounting for anatomical variations [276, 282, 283]. Despite growing use in clinical practice, the role of the rectal boost is not yet validated, and no consensus exists regarding its optimal delivery. To support future recommendations, a systematic review and meta-analysis of rectal boost strategies was conducted to identify factors associated with tumour response [277]. Beyond dose escalation, RT is also explored as an immunotherapy sensitiser within TNT, aiming to enhance CR and facilitate organ preservation. A phase II trial in microsatellite-stable (MSS) LARC was proposed here to evaluate immunotherapy combined with TNT, integrating a primary tumour-only RT boost while delaying pelvic lymph node irradiation to preserve tumour-draining lymph node (TDLN) immune function.

In metastatic CRC, RT is primarily indicated in oligometastatic disease as an alternative to local treatments such as surgery or radiofrequency ablation. Liver metastases, the most frequent metastatic site, can be controlled with stereotactic ablative RT (SABR), though technical challenges arise from lack of tumour visibility on CBCT during image-guided RT and respiratory motion. To overcome these, CBCT-guided oART has been applied to propagate liver target volumes from planning CT to daily CBCT in a margin-based approach [278]. Additionally, mechanically assisted non-invasive ventilation has been investigated to induce stable apnoea's, reducing motion artefacts, improving CBCT quality, and thereby enhancing both image-guided RT and

oART procedures [176-178, 279]. The potential synergy between RT and immunotherapy may also benefit metastatic MSS CRC patients ineligible for local therapy. While preclinical data support this strategy, clinical results remain limited. A better understanding of RT-induced immune modulation in humans is essential to optimise RT-immunotherapy combinations. To this end, a prospective study in MSS CRC patients undergoing preoperative RT before radical surgery has characterised systemic and local immune modifications according to RT dose and target site. Safety and feasibility of this approach have been demonstrated.

## **5.2. Locally advanced rectal cancer**

### **5.2.1. Online-adaptive radiotherapy for dose escalation**

The present work demonstrates the feasibility of CBCT-guided oART in LARC, particularly for delivering a boost to the primary tumour. oART enabled reduction of planning target volume (PTV) margins, thereby allowing higher tumour doses compared to non-adaptive RT without increasing the risk of radiation-induced toxicity. This approach could potentially improve complete response rates [276]. However, these findings remain based on *in silico* data, underscoring the need to confirm clinical feasibility and outcomes relative to conventional non-adaptive RT. A retrospective series of 10 LARC patients treated with long-course chemoradiotherapy (45 Gy to the pelvis with a 54 Gy boost to the primary tumour) using CBCT-guided oART reported a median session time of 22 minutes. With standard PTV margins, adaptive planning achieved significantly improved target coverage

compared to scheduled plans ( $p < 0.05$ ), and three patients achieved clinical complete response after RT alone, without neoadjuvant chemotherapy [195].

Other oART techniques are being evaluated in LARC with the same aims: limiting OAR toxicity and enabling dose escalation to the primary tumour. A phase II trial by Wang et al. tested diagnostic CT-based oART in 13 patients receiving long-course pelvic RT (45 Gy in 25 fractions). Thanks to image quality improvements, PTV margin was reduced from 8 – 10 mm with non-adaptive RT to 5 mm with oART, with further potential down to 3 mm. Reported toxicities were limited to 23.0% Grade 2 gastrointestinal and 7.7% Grade 2 genitourinary events, with no Grade 3 toxicities. However, this study did not explore the role of a primary tumour boost [583]. MRI-guided oART offers direct, real-time tumour visualisation, an advantage over CBCT, but remains less available and resource-intensive. Evidence in LARC is limited, particularly for long-course RT, as most studies have focused on short-course RT (5 fractions), where a boost to the primary tumour remains experimental [347, 397, 428, 584, 585]. All these studies aimed to quantify and reduce PTV margins compared to non-adaptive strategies. In a pilot study by Boeke et al. five LARC patients received MRI-guided oART with 45 Gy to pelvic nodes in 25 fractions, a simultaneous integrated boost to 50 Gy for the primary tumour, and a weekly 3 Gy adaptive boost based on intra-treatment tumour response evaluation. Four patients achieved clinical complete response and one achieved pathological complete response, with acceptable toxicity and restoration of baseline quality of life at 3 months [347].



In December 2024, our institution treated its first LARC patient using CBCT-guided oART with the PTV margins defined in Section 3.1. for our adaptive workflow. Treatment consisted of 45 Gy to the pelvic lymph nodes with a simultaneous integrated boost to 54 Gy for the primary tumour, in line with institutional protocols. Further dose escalation is not currently planned outside the protocol described in Section 3.3 of this work. A retrospective and prospective study will soon be launched to systematically collect data from these oART cases and compare outcomes with non-adaptive RT (National registration number: B4032025000069). Similar investigations are ongoing, such as a pan-cancer trial encompassing LARC and evaluating CBCT-guided oART (NCT06116019). In parallel, a phase III Chinese trial is comparing dose-escalated long-course chemoradiotherapy (via simultaneous or sequential boost, incorporating oART approaches such as CBCT-guided oART) with non-escalated RT in the TNT setting for LARC. Its co-primary endpoints are pathological complete response rate and surgical difficulty (NCT06246344) [586].

### **5.2.2. Optimisation of the online-adaptive radiotherapy workflow**

Further improvements of the CBCT-guided oART workflow should be investigated at multiple levels to optimise treatment quality. oART requires daily delineation of target volumes and OARs, which may be performed by different operators and thus subject to inter-observer variability. In preliminary work, we showed that variability in rectal boost volume

delineation was lower among expert radiation oncologists, while no significant differences were observed between volumes delineated by non-expert radiation oncologists, radiation therapists, and automatically generated contours from an oART-dedicated planning system [283]. These findings underscore the importance of interventions such as consensus guideline development, use of multimodal imaging, and training initiatives to minimise variability [306, 587, 588]. Moreover, automatic oART-generated boost volumes proved reliable compared to non-expert and therapist contours, even without manual correction, and could accelerate the workflow by reducing the most time-consuming step, the manual edition of volumes [194, 313, 314]. Although CBCT-guided oART sessions are shorter ( $\approx 25$  minutes) than MRI-guided sessions ( $\approx 45$  minutes), further reducing adaptation time is essential to enhance patient comfort and limit intrafraction motion of the rectum and adjacent pelvic OARs [194, 195, 428]. Our preliminary data demonstrated that rectal motion increased with session duration and with distance from the anorectal junction, highlighting the need to consider these parameters when planning oART for LARC [282]. In addition, most motion variation occurs between 1 and 18 minutes after reference image acquisition, further supporting strategies to shorten oART session duration [166]. Improvements could target different workflow steps: enhancing autosegmentation model performance to limit manual corrections, reducing CBCT acquisition time, and standardising procedures to increase efficiency [522, 589]. Given that CBCT-guided oART is resource- and time-intensive, identifying which patients benefit most is a key issue. In a retrospective study of 93 LARC patients treated with long- or short-course

RT, Chen et al. evaluated 18 potential predictive factors of oART need, defined as  $\geq 2$  violations of dosimetric constraints when initial plans were recalculated on daily imaging. Multivariate analysis identified high PTV volume, low PTV dose homogeneity index, neoadjuvant RT (as opposite to adjuvant), higher dose per fraction, and high accumulated dose (related to the advancement of the RT treatment) as independent predictors of increased requirement for online adaptation [590].

### **5.2.3. Rationale for a rectal boost**

By demonstrating *in silico* that escalating the dose to 60 Gy for the primary tumour could increase tumour control probability without elevating pelvic toxicity risk, our study provides additional support for performing an external beam RT boost to the rectal tumour [276]. Nevertheless, this boost remains unvalidated despite its widespread use in clinical routine during long-course RT. Two meta-analyses reported high pathological complete response rates with acceptable toxicity for dose escalation  $\geq 60$  Gy in one study and  $\geq 54$  Gy in another, though neither compared patients with versus without dose escalation [163, 591]. Similarly, recent pivotal TNT trials included a primary tumour boost [103, 115]. The benefit of dose escalation is also supported by endorectal brachytherapy studies, which achieved a 3-year organ preservation rate of 81% [121]. Conversely, the RECTAL-BOOST trial showed no improvement in CR rates with a 5 x 3 Gy boost compared to no boost, while reporting transient increases in pain, fatigue, and diarrhoea, as well as temporary deterioration in quality of life [164, 165]. Current

European Society for Medical Oncology (ESMO) guidelines do not clearly recommend an external beam boost for localised rectal cancer [94]. In practice, boost strategies are therefore guided more by institutional protocols than by high-level evidence.

This variability was confirmed by our systematic review of rectal boost practices [277]. Also, an Italian survey in 2021 found that 55.3% (21/38) of RT centres incorporated a boost during long-course RT, with wide heterogeneity in imaging modalities, CTV definition, prescribed dose, and selection criteria for intensification [290]. The novelty of our review lies in its meta-analysis of boost features associated with pathological complete response, providing a foundation for evidence-based recommendations [277]. These results could potentially be considered in the Delphi consensus process currently being conducted by the European Society for Radiotherapy and Oncology (ESTRO) guidelines committee on rectal boost RT.

Based on our findings, we propose:

- Use of IMRT or VMAT instead of 3D conformal RT
- No numerical or anatomical margin added to the GTV for CTV definition
- Preference for simultaneous integrated boost over sequential boost
- Dose escalation above 74 Gy in biologically effective dose (equivalent to >53 Gy in 2 Gy fractions)

In addition to the forthcoming ESTRO guidelines, future trials will clarify the benefit of external beam rectal boosts. A Chinese phase III study

(NCT06246344) is comparing standard long-course RT (50 – 50.4 Gy in 25 – 28 fractions without boost) versus dose-escalated long-course RT (same regimen with a 60 – 65 Gy boost to the primary tumour) in stage II-III LARC [586].

#### **5.2.4. Patient selection for a rectal boost**

A limitation of our meta-analysis is the assumption that all LARC patients should benefit from dose escalation to the primary tumour. Predictive models of pathological complete response based on pre-treatment characteristics could potentially guide patient selection for boost strategies. Over the past year, multiple models have been developed using diverse approaches, including multi-omics (proteomics, whole-exome sequencing, RNA sequencing), radiomics (CT, MRI, PET/CT), and deep learning models integrating clinical data, radiomics, pathology, and endoscopic imaging [592]. Most existing models are radiomics-based and rely predominantly on MRI, with superior predictive performance reported for 3T MRI compared to 1.5T MRI [592, 593]. Another promising approach is the adaptive escalation of RT dose based on tumour response during treatment. This requires repeated imaging for response monitoring, which is feasible with MRI-guided RT. For instance, Boeke et al. combined a simultaneous integrated boost of 50 Gy with a weekly adaptive boost of 3 Gy, delivered until significant tumour shrinkage was observed on daily MRI [347]. Similarly, the THUNDER-2 phase II trial uses daily MRI to calculate an early tumour regression index at the 10th fraction to identify non-responders.

From the 11<sup>th</sup> fraction, these patients receive a total dose of 60.1 Gy to the primary tumour, compared to 55 Gy for early responders. The primary endpoint is a 10% increase in CR rate among early non-responders. While efficacy results are pending, preliminary feasibility and safety data showed no Grade  $\geq 3$  toxicities in the boost group [334].

#### **5.2.5. Other rectal boost modalities**

In addition to long-course RT, rectal boost strategies are also being explored within short-course RT regimens. In the phase I preRADAR trial, a sequential MRI-guided boost is delivered to the primary tumour following conventional 5 x 5 Gy, testing four dose levels ranging from 2 to 5 additional fractions of 5 Gy, with the goal of defining the maximum tolerated dose for future trials aiming to enhance organ preservation [397]. Another phase I study is evaluating simultaneous boost doses of 5 x 6 Gy to 5 x 8 Gy, followed by four consolidation cycles of 5-fluorouracil and oxaliplatin, with pathological complete response as the primary endpoint [405].

Alternative modalities to external beam RT are also being considered for boosting the primary rectal tumour. Endorectal contact x-ray brachytherapy has demonstrated high clinical complete response rates and potential for organ preservation, though it has mainly been studied in less advanced LARC (tumours  $< 3 - 5$  cm, excluding T4) [121, 291]. Protontherapy is under investigation as a means to escalate preoperative rectal RT doses to the primary tumour while reducing exposure of OARs compared to conventional photon therapy, thereby potentially limiting toxicity. To date, however, only

planning studies are available and no clinical data in humans have been reported [352, 594].

#### **5.2.6. Beyond dose escalation: potential of rectal tumour-only irradiation**

Using our CBCT-guided oART workflow, we demonstrated a substantial reduction in PTV margins (3.3 mm antero-posterior, 3.2 mm left-right, and 8.7 mm supero-inferior) compared with non-adaptive RT (14.2 mm antero-posterior, 5.0 mm left-right, and 12.3 mm supero-inferior [276]. This translated into a significant decrease in irradiated volume (123 cm<sup>3</sup> versus 214 cm<sup>3</sup>). When applied to the rectal tumour alone, without prophylactic pelvic RT, this strategy could spare mesorectal lymph nodes, the first tumour-draining relay in rectal cancer. Such an approach aligns with our proposed protocol (Section 3.3) of an initial tumour boost combined with immunotherapy while preserving TDLN integrity.

Preservation of TDLNs could be facilitated through margin-reduction strategies, including CBCT-guided oART, as discussed, or MRI-guided oART. Other approaches include plan libraries, early-treatment imaging for adaptive margin recalculation, or fiducial-based image-guided RT [595, 596]. Although direct comparisons of these methods in terms of lymph node functional preservation are not feasible within our phase II trial, positive results would pave the way for a multicentric study. Such a trial could evaluate different node-sparing strategies across centres, with translational analyses of the immune contexture providing valuable data for comparison.

### **5.2.7. Tumour-draining lymph node sparing: towards a paradigm shift ?**

The addition of immune checkpoint inhibitors to TNT with long-course RT for MSS LARC has been investigated in phase II trials (NECTAR, VOLTAGE, NCT04304209) [74, 152, 597]. These studies reported CR rates ranging from 30% to 45%, while in the only trial with a control group (NCT04304209), the CR rate in the control arm was 26.9%. In our institutional experience, without immune checkpoint inhibitor during long-course RT-based TNT, 55.7% (21/38) of patients achieved an initial CR (17 clinical complete response, 4 pathological complete response). Among these, 4 relapsed locally after non-operative management and required salvage total mesorectal excision, resulting in an organ preservation rate of 34.2% (13/38). These outcomes were similar to those reported in the above-mentioned phase II studies, questioning the clinical benefit of adding immune checkpoint inhibitors, which is not currently recommended. When compared to the impressive efficacy of immune checkpoint inhibitors in dMMR/MSI-H LARC, results in MSS LARC highlight the need for further optimisation of immunotherapy strategies. These modest results are similar to findings in other tumour types, such as head and neck squamous cell carcinoma and non-small cell lung cancer [252, 253]. In the JAVELIN Head and Neck 100 trial, chemoradiotherapy with or without avelumab (anti-PD-L1) showed no significant improvement in progression-free survival [253]. The leading hypothesis for these negative results is that prophylactic RT to TDLNs, required for oncological control, suppresses the RT-immunotherapy synergy that normally occurs within these nodes [253]. As in head and neck



cancer, RT for LARC includes elective pelvic nodal irradiation beyond the primary tumour, encompassing rectal-draining nodes [161, 598]. Preclinical data demonstrate that removal or irradiation of TDLNs reduces response to RT, immune checkpoint inhibitors, and their combination, in parallel with a decrease of CD8<sup>+</sup> T cell tumour infiltration [248, 250, 599]. Following RT-immune checkpoint inhibitors treatment, CCR7<sup>+</sup> dendritic cells migrate to TDLNs to prime CD8<sup>+</sup> T cell responses. Interruption of this migration or destruction of TDLNs abrogates the anti-tumour effect, underscoring the central role of dendritic cells and supporting strategies using dendritic cell-stimulating immunotherapy (such as mitazalimab, a CD40 agonist, Section 3.3.) [599].

Nevertheless, pelvic nodal irradiation reduces local recurrence in LARC and its omission is not currently recommended [94, 98, 161]. Importantly, delaying TDLN irradiation relative to primary tumour RT and immune checkpoint inhibitors initiation has been shown to restore tumour regression [249]. In humans, this concept is being tested in the Neo-CheckRay trial in luminal B breast cancer. In this study, 3 x 8 Gy is delivered to the primary tumour only, sparing axillary lymph nodes even if involved, followed by systemic therapy according to randomisation: chemotherapy alone, chemotherapy with durvalumab (anti-PD-1), or chemotherapy with durvalumab and oleclumab (anti-CD73). Standard surgery and adjuvant therapy then follow [600]. In head and neck cancer, stereotactic ablative RT delivered to the primary tumour while sparing TDLNs, when combined with immunotherapy and followed by delayed TDLN resection, has been shown to increase the proportion of activated CD8<sup>+</sup> T cells in both the primary

tumour and TDLNs compared with untreated patients [251]. To our knowledge, no protocol is currently investigating delayed TDLN RT in combination with immune checkpoint inhibitor for MSS LARC treated with long-course RT. However, growing interest in nodal-sparing strategies is evident. A recent review highlighted multiple trials testing this concept across cancer types, including two Chinese studies in LARC (NCT06507371 and NCT06204094), where TNT combines anti-PD-1 therapy, consolidation chemotherapy, and short-course RT to the primary tumour only, without any TDLN RT [254, 601, 602]. After decades of meticulous efforts to understand lymphatic spread and define target volumes for prophylactic irradiation, we may now be facing a paradigm shift in RT: de-escalating nodal irradiation to preserve immune function of TDLNs, thereby enhancing the efficacy of immunotherapy.

### **5.3. Metastatic colorectal cancer**

#### **5.3.1. Image-guided RT in liver SABR**

In this work, we also evaluated two innovative techniques for liver SABR with direct implications for image-guided RT. The first is CBCT-guided oART, with the objective to avoid percutaneous implantation of radiopaque markers, which can be morbid, for image-guided RT. In oART sessions, target volumes are automatically propagated from the planning CT to the daily CBCT. Even with the best available propagation algorithms, residual errors remain and must be integrated into the PTV margin to prevent geographic miss, which results in larger irradiated volumes compared to non-adaptive RT. However,

by recomputing a new plan based on daily anatomy, target coverage and OAR sparing are improved compared to non-adaptive RT [278]. The next step is clinical implementation, though the optimal propagation strategy we tested was only available for research and has not been incorporated into the current oART software. Applying this workflow in routine clinical practice will require recalculating PTV margins and reassessing the dosimetric impact. In the literature, a retrospective study of 13 patients treated with 5-fraction liver SABR (45 – 60 Gy) using CBCT-guided oART showed significantly improved PTV coverage and reduced maximum doses to OARs compared with scheduled plans ( $p < 0.05$ ), particularly in cases of overlap between PTV and OARs. However, this study still relied on radiopaque markers and used an empirical isotropic PTV margins of 3 – 5 mm [196].

The second innovation concerns the use of mechanically assisted non-invasive ventilation-induced breath-hold (MANIV-BH) to improve CBCT quality in liver SABR. We demonstrated that MANIV-BH enhances CBCT image quality and reduces inter-operator variability in image-guided RT, supporting its use in routine practice, as already implemented in our institution. Moreover, MANIV-BH improves the accuracy of automatic deep learning-based autosegmentation of OARs, reducing the need for manual corrections and optimising the workflow. These results pave the way for broader clinical implementation of CBCT-guided oART in liver SABR [279].

Comparison with MRI-guided RT has been developed in Sections 4.1.4. and 4.2.4. Other strategy used for liver SABR include tracking. As opposite to our proposed workflow using CBCT-guided oART it requires the implantation of

radiopaque markers, which is precisely what we are trying to avoid with our method [603, 604]. The range of the session treatment duration is wide with median values of 107 minutes (range: 36 to 199 minutes), which is much more than what we expected within our markerless CBCT-guided workflow [605]. Tracking techniques does also have to consider uncertainties based on 3 components: system-related tracking and positioning errors ( $< 1$  mm), uncertainties of the correlation between fiducials position and optical tracking system (3.9 mm) and errors related prediction of the target position in the future (approximately 0.5 mm) [604, 606]. When integrating together, these values are similar to the propagation uncertainties quantified in our analysis of the markerless CBCT-guided oART workflow. However, tracking system overcomes the uncertainties associated with tumour motion.

### **5.3.2. Integration of new technologies for liver SABR improvement**

Combining CBCT-guided oART and MANIV-BH for liver SABR has the potential to offer synergistic benefits. Regarding PTV margin reduction, MANIV-BH addresses two sources of uncertainty integrated in the van Herk-based PTV recipe described in our study (Section 4.1. Eq. 4.1.2). First, the margin accounted for tumour motion under free-breathing conditions. By significantly reducing tumour motion, MANIV-BH consequently decreases PTV margin size [178]. Second, since optimal target volume propagation during oART depends on image features, the improved CBCT quality achieved with MANIV-BH could reduce propagation error. However, our

MANIV-BH study did not demonstrate such a reduction compared with free-breathing CBCTs. As discussed, this comparison should be interpreted cautiously, as the free-breathing group differed from that used in other analyses, with CBCT images acquired on a different imaging system [279]. Finally, improved CBCT quality enhances the accuracy of OAR autosegmentation, reducing manual correction time and potentially shortening overall session duration [279].

Although we have not yet implemented MANIV-BH with CBCT-guided oART in clinical practice, this approach warrants further investigation. Notably, other BH strategies during CBCT-oART have already been reported or are under evaluation for upper abdominal and thoracic tumours, including gastric lymphoma treatment at our institution [196, 607-610].

With the recent integration of the HyperSight CBCT solution (Varian, a Siemens Healthineers company), our institution now has access to a technology shown to provide high-quality imaging with reduced artifacts, particularly under BH conditions [522]. As previously discussed, improving image quality in this way could reduce treatment uncertainties, thereby enabling smaller PTV margins and more efficient oART procedures. Furthermore, HyperSight allows CBCT acquisition within only 6 seconds, providing an additional advantage in clinical oART workflow [522].

### **5.3.3. Resource consumption**

Despite the demonstrated benefits, CBCT-guided oART and MANIV-BH raise concerns regarding costs and resource utilisation. In our institution, the CBCT-guided oART system used is the ETHOS platform (Varian, a Siemens Healthineers company). While the exact installation cost is difficult to estimate due to multiple factors, the cost of ETHOS, though higher than that of a conventional linear accelerator, is substantially lower than that of an MRI-guided linear accelerator. This makes CBCT-guided oART a potentially more affordable alternative to MRI-guided RT [611]. Nevertheless, significant human and time resources are required for implementation. Stanley et al. reported a period of 11 months from project initiation to first patient treatment, including training of the entire radiation oncology team [612]. In terms of per-fraction cost, CBCT-oART is associated with longer treatment sessions and increased human resources involvement, resulting in an average additional cost of USD 103.58 per session compared with conventional image-guided RT sessions, based on a retrospective series of 21 pelvic cancer patients [613]. However, true cost-effectiveness remains complex to define, given the potential long-term benefits of oART in reducing RT-induced toxicities and their associated management costs.

Compared with non-adaptive workflows, oART adds approximately 15 – 20 minutes per session [612-614]. A learning curve effect has been observed at both patient and institutional levels, with initial sessions being longer than subsequent ones. This highlights the importance of structured training, which can be facilitated by the ETHOS emulator provided by the vendor [613,

614]. Efforts to reduce time consumption should particularly target the delineation of target volumes and OARs, consistently reported as the most time-intensive step [313, 314, 525, 615]. Advances in auto-segmentation models, coupled with tools such as uncertainty quantification, are expected to reduce this time [589, 616].

Regarding human resources, CBCT-guided oART typically requires the presence of a radiation oncologist for validation of target volumes and plan selection, as mandated by the Belgian legislation [192, 617, 618]. Transitioning toward radiotherapy technologist-led workflows could reduce this dependency. A planning study involving 34 prostate cancer patients compared workflows based on the number of automatically delineated influencers (OARs guiding target propagation). It showed that increasing the number of influencers with manual correction by radiotherapy technologists reduced the need for radiation oncologist intervention in target volume editing, leading to comparable total session durations despite increased influencer review time. The workflow with the highest number of influencers required, therefore, less oncologist involvement [617]. Expanding radiotherapy technologist responsibilities beyond influencer editing to include target verification and plan evaluation may further speed up processes and reduce oncologist supervision [192, 619]. Decision-support tools such as traffic-light protocols, combined with a culture of trust, respect, and effective communication, can reinforce radiotherapy technologist involvement and facilitate task delegation [619]. Several groups have already clinically implemented full radiotherapy technologist-led

workflows, significantly reducing the required involvement of radiation oncologists [194, 620, 621].

Previous reports, including our own, have demonstrated the feasibility and safety of implementing MANIV-BH during RT sessions [176-179, 529]. However, to our knowledge, we remain the only team to have integrated this technique into routine clinical practice, limiting direct comparison with published data. The required equipment includes two respirators (one for planning and one for treatment), connection hardware, a monitoring screen in the control room, and a respiratory mask for each patient. These costs are relatively minor at the scale of a RT department, particularly when compared with those of linear accelerators.

In terms of human resources, MANIV-BH planning CTs in our department are acquired under the supervision of a physiotherapist specialised in respiratory function, who trains patients in MANIV-BH and sets ventilation parameters (e.g., exhalation duration, inspiratory pressure). Initially, patient training was conducted in an additional one-hour session scheduled on a day separate from RT planning. With increasing experience and optimisation, training is now incorporated into the planning CT session itself. The original 90-minute time slot has been reduced to 60 minutes, similar to the duration of 4D-CT planning with audio-coaching. In our initial analysis, the average duration of a MANIV-BH RT session for liver or lung SABR was 53.1 minutes. With clinical implementation, the timeslot has decreased to 40 minutes, with further reductions to 30 minutes for the first session and 20 minutes for subsequent sessions anticipated in the near future. [178]. MANIV-BH



sessions are now conducted exclusively by radiation therapists trained in the technique, with physiotherapists no longer required and radiation oncologist supervision needed only in a minority of challenging cases. Together with their expanding role in oART processes, this underscores the evolving responsibilities of radiation therapists and their growing importance in the future of RT [619].

#### **5.3.4. RT-mediated immune reaction in CRC**

The most ambitious part of this work was the prospective interventional study in which MSS CRC lesions were preoperatively irradiated to characterise the impact of RT dose and anatomical site on the tumour immune microenvironment. However, recruitment was lower than expected. The main reason was patient refusal to participate. Compared with standard-of-care management, the protocol required four additional hospital visits on separate days, which was time-consuming. Moreover, most patients had never undergone RT, which is not part of the recommended treatment in this setting and is often perceived as unpleasant or harmful (e.g., burns). These factors likely generated anxiety and reduced patients' willingness to participate in such a study [622]. Despite the motivation of contributing to scientific progress, the personal benefit of participation appeared limited relative to the potential RT-related adverse effects, even if a beneficial immune activation might have occurred. The second major cause of exclusion was the short interval between the multidisciplinary tumour board decision for surgery and the scheduled operation. Within this

narrow window, we had to conduct a research consultation, obtain informed consent, perform RT planning, deliver RT, and respect a 4 – 8-day interval between RT completion and surgery. This constraint improved over time as stakeholders became more aware of study requirements. To increase the recruitment, a multicentric strategy was attempted. Although the protocol was approved by the local ethics committee, implementation at external centres proved logistically challenging, given the complexity of experimental RT delivery and sample collection. Consequently, recruitment was ultimately restricted to Cliniques Universitaires Saint-Luc.

The second major limitation of this trial relates to heterogeneity at multiple levels (Table A10.2). There was marked interpatient heterogeneity in both disease characteristics and prior treatments. For example, this was evident in the tumoral mutational landscape, with 6 of 17 patients harbouring *KRAS* mutations. In addition, treatment history varied widely: some patients, particularly those without metastases, were treatment-naïve, whereas others with liver metastases had received multiple lines of systemic and local therapy. Consequently, the tumour immune microenvironment at baseline was likely shaped by these prior systemic therapies before the experimental RT initiation [623]. The tumour samples also reflected this heterogeneity, with some showing moderate treatment response and residual viable tumour, while others exhibited extensive necrosis, complicating downstream analyses. Finally, as is often the case in clinical practice, variability in sample collection and storage conditions across patients may have further contributed to inconsistent results.

Our initial aim was to identify consistent and strong signatures of RT-induced modulation of the tumour immune microenvironment that could be exploited for pharmacological targeting in novel RT-immunotherapy strategies for metastatic MSS CRC. As always, the biological reality is more complex, which limited our findings to a few signals warranting further study. Tumour RNA sequencing revealed activation of immune pathways including, among others, IFN- $\gamma$ , IFN- $\alpha$ , and TNF- $\alpha$  response, inflammatory response, interleukin-6/JAK/STAT3, and interleukin-2/STAT5 signalling, which were not confirmed in plasma proteomics. This discrepancy may be explained by the low abundance of cytokines in plasma, which are difficult to detect using mass spectrometry but could be captured by more sensitive techniques such as immunoassays (e.g., ELISA, western blot) or ultrasensitive technologies [624]. Flow cytometry analyses suggested an increase in a circulating CD4<sup>+</sup> T-cell subpopulation, referred to as terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells, following RT. At present, limited data are available regarding the role of these cells in antitumour immunity. However, in viral infections, CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells have been shown to exert direct cytotoxic activity [61, 569]. Further characterization of this subset is required to clarify its contribution to RT-induced immune modulation and to determine whether the observed increase in terminally differentiated effector memory RA<sup>+</sup> (TEMRA) cells may account for the modest increase in TCR clonality detected after RT using TCR sequencing. In tumour samples, however, the RNA deconvolution model used was unable to detect this subtype, and our multiplex immunofluorescence panel was designed to

identified other T cell populations described as playing a role in the RT-induced anti-tumour immunity [25, 157]. A new potential multiplex panel allowing CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> (TEMRA) identification should include anti-CD3 for T cell, anti-CD8 for helper T cells (CD8 negative), anti-CCR7 and anti-CD45RO for terminally differentiated effector memory RA<sup>+</sup> (TEMRA), and anti-GPR56 to assess their cytotoxic potential [61, 625]. Additionally, in peripheral blood, increased concentrations of p53 pathway-related proteins were observed following irradiation. This finding may reflect radiotherapy-induced DNA damage, leading to activation of the p53 pathway and the subsequent release of exosomes enriched in p53-regulated proteins that are detectable in the circulation [200, 201]. Advanced biomolecular approaches, though resource-intensive, may be required to precisely characterise the phenotype of some cells of interest (single-cell RNA sequencing) or the spatial relationship between immune subsets and tumour cells (spatial transcriptomic).

MSS colorectal cancers are characterised by a low to moderate tumoral mutation burden. Furthermore, an important part of these mutations affects only a single amino acid, leading to the production of poorly immunogenic and low diversity neoantigens. Therefore, these tumours less able to trigger a strong antitumoral response with a poor infiltration of immune cells in their tumour immune microenvironment and poor response to immune checkpoint inhibitors [46, 626]. By contrast, MSI-H tumours have a high mutational burden with frequent frameshift mutations, which completely change the protein sequence, triggering strong immune infiltration, which

make these tumours sensitive to immune checkpoint inhibitors [44-46, 626]. By directly killing tumour cells, RT induced the release of neoantigen from colorectal. A higher amount of neoantigens are expected in irradiated tumours compared to non-irradiated one. Furthermore, RT has been proven in preclinical models to generate new neoantigens and increase their diversity, which is more associated to immune infiltration and response to immunotherapy [208]. Our TCRseq results demonstrate only a moderate increase of the clonotypic response after irradiation and no change regarding tumoral lymphocyte infiltration after irradiation. Therefore, it can be hypothesised that the increase of neoantigen amount and diversity is not important enough to reach immunogenic potential of MSI tumours, which are importantly infiltrated by immune cells. To further enhance immunogenicity potentially leading to immune responses, other strategies are currently under investigation including neoantigen-based therapeutic vaccines (e.g. peptide-based, nucleic acid-based, or dendritic vaccines) and adoptive cell therapies (e.g. CAR-T, tumour-infiltrating lymphocytes) [627].

Effect of RT on blood vasculature can also explain why the immune infiltration is not significantly modified after irradiation in our sample. Indeed, immune cell migration can be facilitated by low-dose RT (< 5 Gy), which improves blood vessel permeability [210, 213, 214]. On the other hand, higher RT dose may lead to blood vessel damage and restrict tumoral infiltration by immune cells [210, 628]. While, in preclinical models, a dose of 8 Gy seems to do not impact de tumour perfusion, giving multiple 8 Gy fractions as in our protocol could potentially restrict perfusion and therefore immune cell migration [211, 215]. This effect is compounded by the fact that

CRC have an anarchic vascular architecture and that the endothelial cells associated with tumours are characterised by a downregulation of leukocyte adhesion molecules, inhibition of chemotaxis, and inhibition of immune cells activation [629]. By the destruction of the tumour vasculature, RT can also increase the hypoxia associated with CRC tumours. This hypoxia can further reinforce lack of tumour immunogenicity by creating an immunosuppressive microenvironment characterised by the recruitment of Tregs, M2 macrophage polarisation, myeloid-derived suppressive cell and immunosuppressive dendritic cell activation, and by the reduced activation of effector T cells by the hypoxia-induced factor 1 (HIF-1) [630].

Although multiple biomolecular techniques were applied to characterise the tumour immune microenvironment of the included tumours, none could reliably predict response to systemic therapy or other external interventions. Tumour-derived organoids represent a promising approach to evaluate tumour response to ex vivo interventions [631]. In a preliminary work conducted in collaboration with another laboratory of the same institution, we attempted to generate organoids from irradiated tumour samples and from healthy tissue. The project was discontinued early, as only viable organoids were obtained from healthy tissue, while none could be derived from irradiated tumour. This suggests that RT may eliminate stem cells and thereby reduce the proliferative potential required for organoid development (Figure 5.3.1) [632]. As this is an emerging strategy, further methodological improvements are anticipated. For example, Xue et al. reported a 62.3% success rate in establishing organoids from LARC patients treated with neoadjuvant chemoradiotherapy [633]. Irradiated organoids

are also being increasingly used to predict oncological treatment response or RT-induced toxicity, particularly in CRC [634, 635].

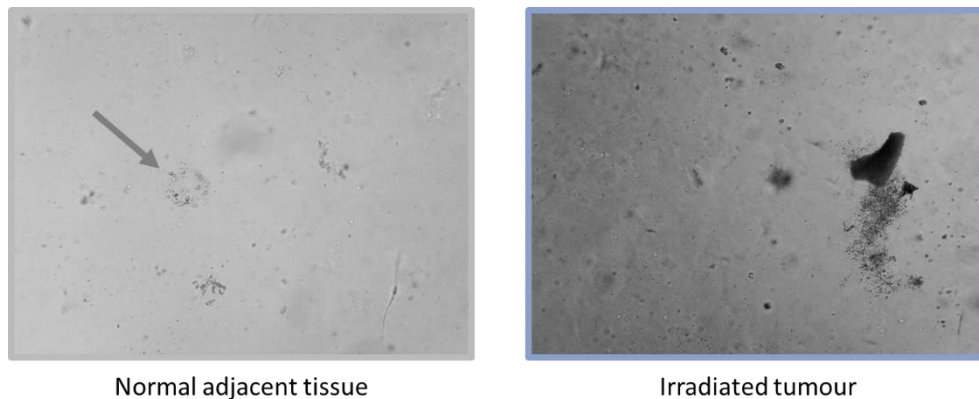


*Figure 5.3.1: Attempts to generate organoid models from irradiated tumours were unsuccessful, as no viable structures suitable for downstream analyses could be established. In contrast, organoids derived from non-irradiated healthy tissue proliferated successfully (dark grey arrow).*

### **5.3.5. Future clinical trials**

The completion of this project has enabled the development of CBCT-guided oART workflows for abdominal and pelvic tumours, the management of a prospective study with biological sample collection investigating preoperative RT, and the exploration of biomolecular techniques for tumour immune microenvironment characterisation. These experiences will serve as a basis for developing protocols for future studies and carrying them out successfully, two of which are currently being developed.

The first protocol will prospectively include all patients undergoing CBCT-guided oART at Cliniques Universitaires Saint-Luc, regardless of tumour type

or RT site. A comparative non-adaptive cohort will be enrolled to assess differences in clinical outcomes between adaptive and non-adaptive treatments. The primary endpoint is acute toxicity difference, defined as RT-induced toxicity occurring during treatment and up to 3 months post-RT, assessed using CTCAE v5.0, patient-reported outcome measures, and validated quality-of-life questionnaires [539]. Secondary endpoints will evaluate both general and cancer-specific oncological outcomes. Clinical, imaging, and RT data will be collected to refine or develop new oART workflows with the ultimate goal of individualising treatment. No fixed sample size has been set, as this study is designed as a continuously evolving prospective registry. Ethical approval has been obtained by the committees of Cliniques Universitaires Saint-Luc (National registration number: B4032025000069), with patient inclusion to start shortly.

The second protocol, described in Section 3.3., explores an innovative strategy in which RT to TDLNs is delayed relative to RT to the primary tumour, aiming to enhance the efficacy of concomitant immunotherapy and increase complete response rates following total neoadjuvant therapy in MSS LARC. Compared with the prospective study described in Section 4.3., this protocol is expected to yield more concrete results. The potential benefits of higher complete response rates and organ preservation within a non-operative management strategy are expected to motivate patient participation, in contrast to the limited individual benefit of the prospective study in Section 4.3., where irradiated MSS CRC lesions were subsequently resected. However, the proposed experimental strategy of combining delayed irradiation of tumour-draining lymph nodes with primary tumour RT



and immunotherapy would not allow the distinction between the effects of the individual contributions of these treatment components when added to standard total neoadjuvant therapy. Moreover, dose escalation to the primary tumour and the addition of immunotherapy constitute a treatment intensification that may increase therapy-related toxicity. Although organ preservation through avoidance of total mesorectal excision could be viewed as a form of treatment de-escalation, this benefit cannot be guaranteed. In other malignancies, such as non-small cell lung cancer and oesophageal cancer, RT dose escalation can be detrimental due to increased toxicity without improvement of oncological outcomes, and current trends favour dose de-escalation strategies, as exemplified by anal canal cancer in the PLATO-ACT4 protocol [636-638]. Importantly, evaluating a strategy limited to primary tumour RT combined with immunotherapy alone would not be ethical in locally advanced rectal cancer, given the well-established benefit of neoadjuvant chemotherapy and pelvic RT on key oncological outcomes [100, 115]. However, total neoadjuvant treatment is not required for all rectal cancers, particularly tumours of the upper rectum, and is not indicated for locally advanced colon cancer, for which upfront surgery remains the standard of care [7, 94]. Upper rectal and sigmoid tumours, where RT may be feasible given acceptable expected tumour motion, could therefore represent an appropriate model to evaluate this approach. Within a “window-of-opportunity” framework between multidisciplinary tumour board decision and scheduled surgery, a short neoadjuvant sequence could be administered with early response assessment guiding surgical management. In this setting, hypofractionated RT to the primary tumour

followed by two cycles of immunotherapy could be delivered over a limited timeframe. Early tumour response assessment using colonoscopy with biopsy, magnetic resonance imaging, and digital rectal examination (when feasible) would inform the decision to proceed with surgery, while simultaneously providing biological samples and imaging data for translational analyses.



## Chapter 6



# Conclusions

Currently, the main challenge in locally advanced rectal cancer (LARC) is to improve patient quality of life through non-operative management with active surveillance within an organ preservation strategy. Radiotherapy (RT) dose escalation to the primary tumour is a potential approach to improve clinical complete response rate. In this work, a cone-beam computed tomography (CBCT)-guided online adaptive RT (oART) workflow for rectal boost was developed, allowing substantial reduction of irradiated volume while maintaining pelvic toxicity risk at levels comparable to non-adaptive RT without dose escalation. However, the role of rectal boost in LARC remains debated and is not included in current guidelines, despite widespread clinical use. The systematic review conducted here revealed major inter-team heterogeneity in boost implementation, highlighting the need for standardised recommendations. Based on meta-analysis, these recommendations could include intensity-modulated RT/volumetric modulated arc therapy rather than three-dimensional RT, no numerical or anatomical clinical target volume margins, simultaneous over sequential boost, and >74 Gy in biologically effective dose. To further improve complete response rates and expand eligibility for non-operative management, a phase II trial protocol was proposed, investigation an innovative total neoadjuvant therapy: RT to the rectal tumour only combined with immunotherapy, followed by neoadjuvant chemotherapy and immunotherapy, and finally pelvic nodal RT. Delaying tumour draining lymph node irradiation is expected to preserve immunotherapy action taking place within these lymphatic sites.

In metastatic colorectal cancer (CRC), liver stereotactic ablative RT is challenging due to poor tumour visibility on daily CBCT and tumour motion related to breathing. In this work, CBCT-guided oART has been shown to allow avoidance of percutaneous radiopaque marker implantation by automatically propagating target volumes to daily CBCT. Although this increases planning target volume size due to propagation error, daily adaptive dosimetry improves target coverage and organ-at-risk sparing compared with non-adaptive RT. In addition to its ability to manage respiratory motion, mechanically assisted non-invasive ventilation was evaluated here for its benefits in image-guided RT. This technique allows the acquisition of better quality CBCTs, the reduction of inter-operator variability in image-guided RT and the performance improvement of organ-at-risk autosegmentation. Finally, in a prospective study of microsatellite-stable CRC patients undergoing surgery, preoperative RT was performed and biological samples were acquired to investigate the RT impact on systemic and local immune environment according to the RT dose and the irradiation site. Feasibility and safety were confirmed. In the blood, RT induced an increase in CD4<sup>+</sup> terminally differentiated effector memory RA<sup>+</sup> cells without significant changes in proteomic profile. Tumour analysis after moderate-dose RT (3 x 8 Gy) showed no clear modification in immune cell infiltration but revealed differential activation of immune pathways suggesting a balance between immunosuppressive and anti-tumour mechanisms. Due to limited recruitment, conclusions for low-dose lesions (3 x 1-2 Gy), non-irradiated lesions in an irradiated patient, non-hepatic metastases, and primary tumours remain exploratory. Future work should further

characterise the immune mechanisms identified here to enable rational RT-immunotherapy combinations aimed at improving outcomes in microsatellite stable CRC patients.

# Chapter 7

## Appendix



## **7.1. Appendix 1: Implications of the organ-specific immune environment for immune priming effect of radiotherapy in metastatic setting.**

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## **Abstract**

With the development of immune checkpoint inhibitors, the tumour immune microenvironment has been increasingly considered to improve cancer management. The tumour immune microenvironment of metastatic lesions is strongly influenced by the underlying immune contexture of the organ in which they are located. The metastatic location itself appears to be an important prognostic factor in predicting outcomes after immune checkpoint inhibitor treatment in cancer patients. Patients with liver metastases are less likely to respond to immune checkpoint inhibitors than patients with metastases in other organs, likely due to variations in the metastatic tumour immune microenvironment. Combining additional treatment modalities is an option to overcome this resistance. Radiotherapy (RT) and immune checkpoint inhibitors have been investigated together as an option to treat various metastatic cancers. RT can induce a local and systemic immune reaction, which can promote the patient's response to immune checkpoint inhibitors. Here, we review the differential impact of the tumour immune microenvironment according to metastatic location. We also explore how RT-induced tumour immune microenvironment modifications could be modulated to improve outcomes of RT-immune checkpoint inhibitor combinations.

### **7.1.1. Introduction**

Due to recent advances in the understanding of cancer, tumoral cells are no longer considered the only target for treatment. The tumour microenvironment also plays an important role in the disease process and response to treatment. A growing body of evidence highlights the significant role of tumour-infiltrating immune cells, which are directly involved in immune destruction evasion, a new cancer hallmark [639]. Targeting immune evasion has led to one of the most significant advances in cancer treatment, namely immunotherapy. The prognosis for both locally advanced and metastatic tumours is significantly improved by inhibiting immunological checkpoints that suppress antitumoral immune responses, such as programmed cell death (PD-1), its ligand PD-L1, or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). However, the effects of immune checkpoint inhibitor differ considerably across tumour histology. Excellent outcomes have been demonstrated for non-small cell lung cancer, melanoma, and colorectal cancer (CRC) with deficient DNA mismatch repair microsatellite instability–high (dMMR/MSI-H). However, results for other primary cancers, such as prostate cancer, hormone-sensitive breast cancer, and CRC with microsatellite stability (MSS) have been less promising [268, 640-643]. One hypothesis for this response heterogeneity is that it is highly dependent on the degree of immune cells' infiltration in the tumour immune microenvironment [644]. Local tumour immune microenvironment could also have a systemic impact on immune checkpoint inhibitors' response in a metastatic situation [645, 646]. Therefore, researchers have investigated the possibility of increasing tumour immune microenvironment infiltration by

immune cells to induce a response to immunotherapy in immune checkpoint inhibitor-insensitive tumours [533, 644]. Given the local and systemic immunological responses brought on by radiation, combining immune checkpoint inhibitors with radiotherapy (RT) may overcome response resistance [533]. Tumour response outside the RT field demonstrates the immunogenic cell death mediated by RT (known as the “abscopal effect”) [203, 204, 647]. Nevertheless, this effect is uncommon and might be influenced by tumour immune microenvironment characteristics [648, 649]. In this review, we review variations in the metastatic tumour immune microenvironment according to the host organ and its impact on immune checkpoint inhibitor outcomes. We also discuss the consequences of RT-induced modifications on the tumour immune microenvironment and the systemic immune system to guide future development of immunotherapy-RT combinations in currently non-responding tumours.

### **7.1.2. Metastatic Location Matters**

The immune microenvironment varies between organs and is influenced by multiple factors, including exposure to external antigens, organ function, and anatomy. Thus, the tumour immune microenvironment of metastases differs depending on hosting organs. This variation may account for the different responses to immune checkpoint inhibitors observed in patients with a similar primary tumour in different metastatic locations. Of the most frequent metastatic locations, the presence of secondary lesions in the liver

appears to have the most detrimental impact on the prognosis of patients receiving immune checkpoint inhibitors.

#### *7.1.2.1. Liver Metastases*

The liver is exposed to many antigens and microbial products that pass through the portal system. These molecules are removed from the blood system by liver cells to prevent an inflammatory reaction [76, 650, 651]. A wide variety of immune cells exist in the liver sinusoids, including Kupffer cells, which produce caspase-3 mediated signals to induce T cells' apoptosis (such as Fas-ligand and TNF-receptor apoptosis-inducing ligand). T cell function is further inhibited by immunosuppressive molecules, including PD-L1, interleukin-10, and transforming growth factor (TGF)- $\beta$ , which are secreted by liver-specific antigen-presenting cells (such as Kupffer cells, hepatic stellate cells, and sinusoidal endothelial cells). In addition, sinusoidal endothelial cells and natural killer cells stimulate immunosuppressive regulatory T cells (Tregs), which inhibit the action of T cells. However, this state of tolerance favours the establishment and growth of metastases in the liver [76, 650-652]. Kupffer cells, neutrophils, and sinusoidal endothelial cells also contribute to the creation of the liver pre-metastatic niche by secreting adhesion molecules that are recognised by cancer cell receptors.

Once established, liver metastases (among frequent metastatic locations, the liver metastases) induce immune modifications that lead to a "systemic immunosuppressive state". This phenomenon has been inferred from preclinical models that indicate that liver metastasis resection decreases

systemic immunosuppression [76]. Studies in mice have revealed a reinforcement of Fas-ligand-mediated T cell apoptosis by CD11b<sup>+</sup> myeloid-derived suppressor cells, which were particularly enriched in the liver metastasis tumour immune microenvironment [538]. In addition, mice with liver metastasis had increased activation of Tregs (CTLA-4, PD-1, and high inducible co-stimulatory molecule), which is known to contribute to immunosuppression (Figure A1.1) [653]. A clinical consequence of this immunosuppressive state is poor prognosis for patients with liver metastasis, which has been observed in several cancers regardless of systematic treatment [645, 646, 654-661].

Particularly regarding immune checkpoint inhibitors, liver metastasis-mediated immunosuppression has a significant impact on oncological outcomes [538, 645, 661-668]. A retrospective study by Tumei and colleagues included two cohorts of 223 and 113 metastatic melanoma patients, respectively, and one cohort of 165 metastatic non-small cell lung cancer patients treated with pembrolizumab monotherapy [645]. Compared to lung and brain metastases, liver metastases were found to be an independent poor prognostic factor significantly associated with a shorter progression-free survival in all three cohorts. The lower density of CD8<sup>+</sup> T cells observed in liver metastasis tumour immune microenvironment compared to that observed in non-liver metastases may account for this difference. Moreover, non-liver metastases from patients with synchronous liver metastasis had a lower CD8<sup>+</sup> cells density than those from patients with no liver metastasis [645]. In a study by Yu and colleagues, liver metastasis were also associated with a reduced objective response rate and a reduced

overall survival in melanoma (n = 182) and non-small cell lung cancer (n = 279) patients treated with immune checkpoint inhibitors, compared to those of patients with brain or lung metastases. Interestingly, this association was not observed for targeted therapy in melanoma patients or chemotherapy in non-small cell lung cancer patients [538]. A retrospective report by Osorio and colleagues evaluated metastatic non-small cell lung cancer (n = 214) and inducible co-stimulatory molecule carcinoma (n = 78) patients treated with PD-1 inhibitors and found that the liver was the most frequent site of progression in both cohorts (non-small cell lung cancer: 24.0%; inducible co-stimulatory molecule carcinoma: 29.7%). Although the response to PD-1 blockade was uniform among synchronous metastases, progression was found to be heterogeneous. The authors hypothesised that the immunotolerance of the tumour immune microenvironment in liver metastasis explained why the liver was the first progression site following PD-1 blockade therapy [668].

The negative impact of liver metastasis has been observed even in cancers with a poor intrinsic response to immune checkpoint inhibitors. Wang and colleagues conducted a study of 95 MSS CRC patients treated with PD-(L)1 immunotherapy. Patients without liver metastasis had a superior objective response rate (19.5%) and progression-free survival (4.0 months) compared to those with liver metastasis, for whom no objective response or increased progression-free survival (1.5 months) were demonstrated [646, 668]. Similarly, a reduced response rate was observed in patients with liver metastasis who received anti-PD-1 and anti-CTLA-4 combination therapy. In a retrospective cohort of 140 metastatic melanoma patients who received

first-line treatment with dual immune checkpoint inhibitors, the best outcomes (objective response rate and progression-free survival) were found in patients with lung metastases, whereas comparatively poor results (objective response rate, progression-free survival, and overall survival) were observed in patients with liver metastasis [669].

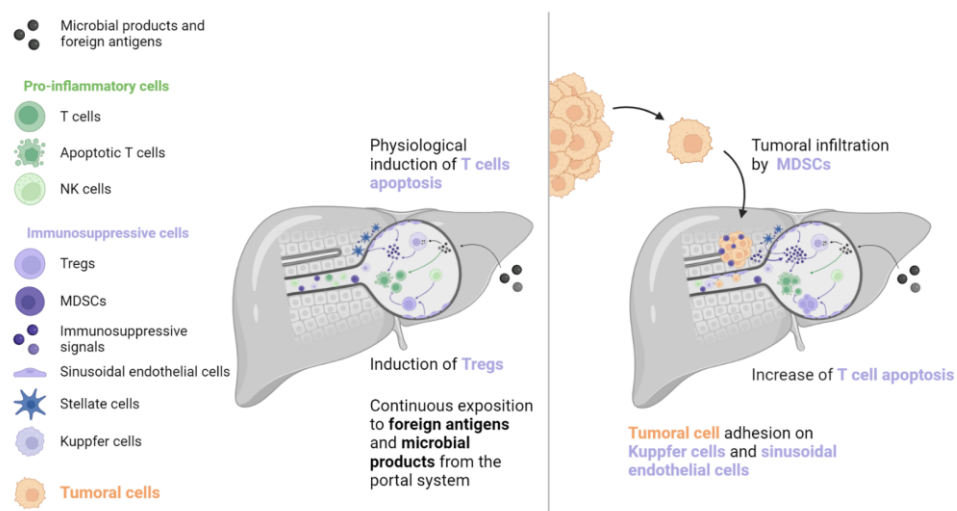


Figure A1.1: To prevent immune system overactivation, the liver has tolerogenic mechanisms that are primarily mediated by the induction of T cell apoptosis. When a tumour is present, this pathway is strengthened by molecular signals originating from tumoral and tumour immune microenvironment cells (such as Myeloid-derived suppressor cells, Kupffer cells, sinusoidal endothelial cells, and stellate cells). T cell inhibition and depletion may explain the low efficacy of immune checkpoint inhibitors in patients with liver metastases. MDSC: Myeloid-derived suppressive cell, NK: Natural killer, Treg: Regulator T cell.



#### 7.1.2.2. *Non-liver Metastases*

The lungs, like the liver, are a common metastatic site because they are also exposed to a variety of foreign molecules (e.g., tobacco, traffic pollution, inhaled micro-organisms). Thus, tolerance mechanisms are necessary to prevent inappropriate immune reactions. In the lungs, such mechanisms include epithelial cells, airway macrophages, mucociliary action, and commensal microbioma [670]. These mechanisms have the potential to favour a pre-metastatic niche formation, often due to overexpression of the cytokine CCL2, which is derived from the primary tumour and leads resident macrophages and endothelial cells to produce endogenous Toll-like receptor 4 ligands such as S100A8 and SAA3. These effectors facilitate the recruitment of Myeloid-derived suppressor cells and increase vascular permeability, supporting tumoral cell implantation [671]. In addition,  $\gamma\delta$ T cells account for up to 20% of T cells in the lung ecosystem and may promote tumour growth by inducing neoangiogenesis and evasion by restricting the action of Antigen-presenting cells, recruiting Myeloid-derived suppressor cells, and secreting immunosuppressive molecules such as interleukin-4, interleukin-10, and TGF- $\beta$  (Figure A1.2) [672].

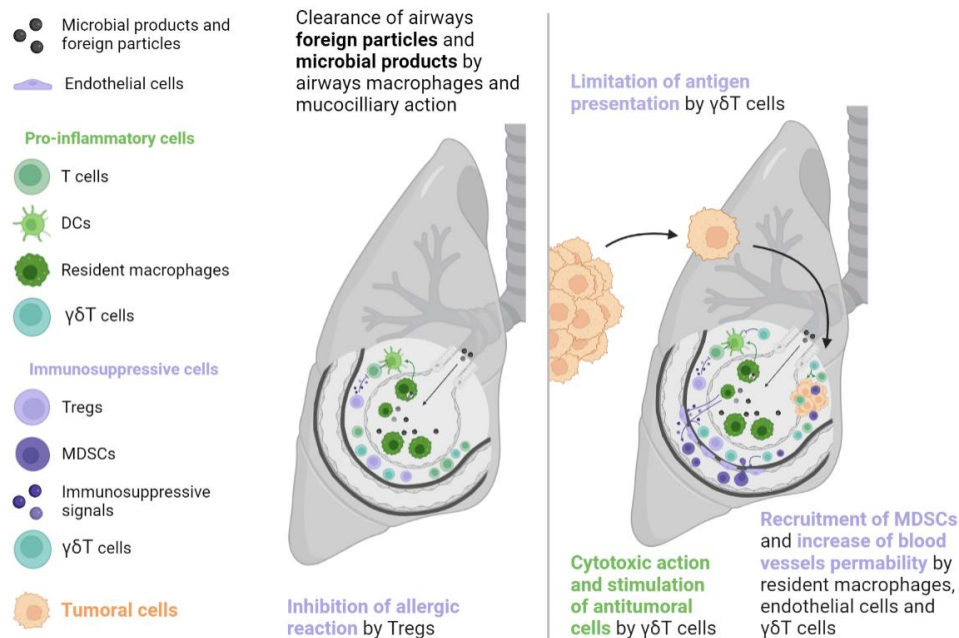


Figure A1.2: Tolerogenic mechanisms in the lungs involve less T cell apoptosis than those in the liver. In the presence of a tumour, a particular lymphocyte population called  $\gamma\delta$ T cells can have ambiguous effects on tumour development. These cells exert a direct cytotoxic action and an indirect antitumoral action via the stimulation of T cells. However, they can also facilitate tumour development through the secretion of immunosuppressive signals, recruitment of immunosuppressive cells, promotion of neoangiogenesis, and limitation of antigen presentation. DC: Dendritic cell, MDSC: Myeloid-derived suppressive cell, Treg: Regulator T cell.

Bone is another common metastatic site in various solid malignancies, particularly prostate cancer, non-small cell lung cancer, renal cell carcinoma, and breast cancer [673]. The bone environment is considered immune tolerant due to the abundance of Tregs and myeloid-derived suppressor cells and the lack of natural killer and cytotoxic T cells [674-676]. Primary tumour

cells can generate systemic molecular signals that interact with osteoblasts and osteoclasts to promote angiogenesis, cancer cell adhesion, and bone resorption. The combination of these actions generates a pre-metastatic bone niche (Figure A1.3) [675, 677].

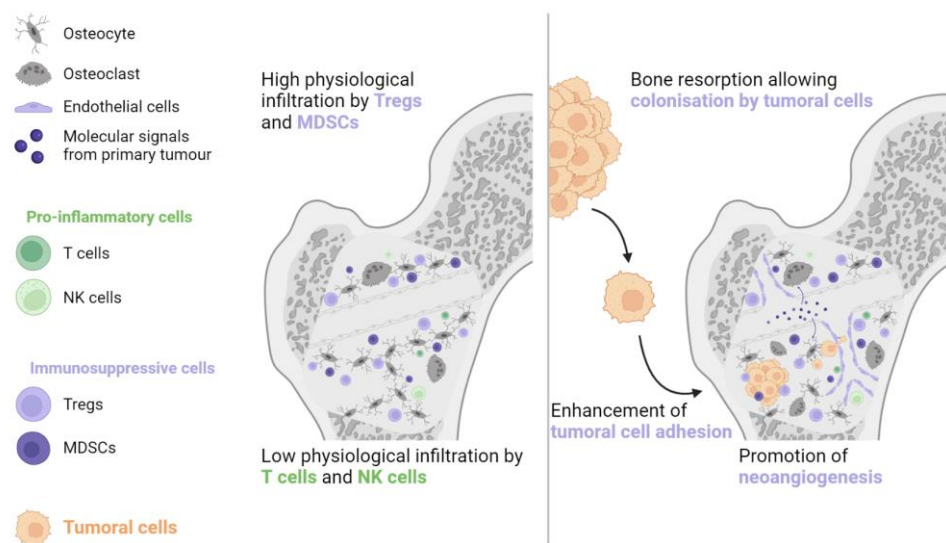


*Figure A1.3: The physiological abundance of Tregs and Myeloid-derived suppressor cells, combined with the paucity of anti-tumoral immune cells, is the main reason why bones are frequently invaded by metastatic lesions. The primary tumour generates molecular signals, which contribute to the formation of a pre-metastatic niche by increasing bone resorption, stimulating neoangiogenesis, and enhancing tumour cell adhesion. MDSC: Myeloid-derived suppressive cell, NK: Natural killer, Treg: Regulator T cell.*

For a long time, the brain was considered an immune-depleted organ due to the presence of immunosuppressive molecules and low T cell infiltration in patients with primary brain tumours [678-680]. However, lymphatic vessels have been recently found in regular brain sinuses, transporting immune cells

and molecules to the cervical lymph nodes, the first lymphatic relay [679]. A large population of immune cells has also been found in the dura and meninges around the brain, coming through channels directly from the skull bone marrow [681]. Additionally, the microglia, a specialised population of resident macrophages in the brain, is considered the key responder to all types of brain aggressions. It phagocytoses cellular wastes, migrates to the site of disease, secretes pro-inflammatory factors such as interleukin-1 $\beta$  and tumour-necrosis factor (TNF)- $\alpha$ , and has a direct cytotoxic effect [678, 682]. However, prolonged microglial activation induces chronic inflammation and destruction of normal brain cells (astrocytes and neurons), which creates a local environment that favours metastatic invasion (Figure A1.4) [678, 682, 683]. Although patients with brain metastases are usually excluded from pivotal immune checkpoint inhibitors trials, retrospective data from non-small cell lung cancer, renal cell carcinoma, and melanoma patients suggest that brain metastases are not a prognostic factor for poor objective response rate or overall survival [684-687].

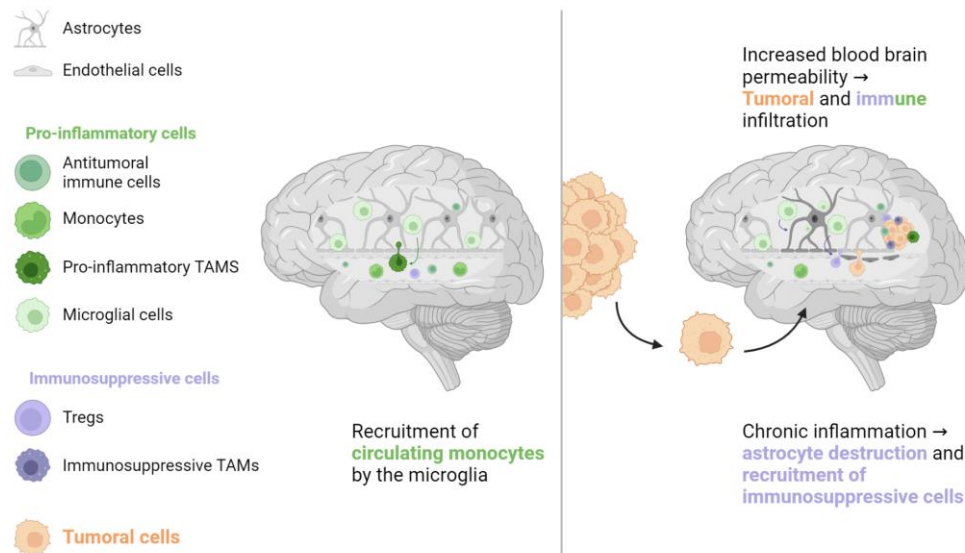


*Figure A1.4: The brain has its own population of macrophages, the microglia. These cells, the main responders to brain injury or aggression, stimulate the recruitment of immune cells, mainly monocytes, from systemic circulation. In the case of chronic inflammation, microglial cells can directly damage normal brain cells such as astrocytes, which are crucial for preserving the blood-brain barrier. Increased blood-brain barrier permeability can promote tumour cell migration and recruitment of immune cells, some of which have an immunosuppressive phenotype that favours tumour cell implantation. TAM: Tumour-associated macrophage, Treg: Regulator T cell.*

Studies that have examined immune checkpoint inhibitors in the metastatic setting do not report lung, bone, or brain metastases as an independent poor prognostic factor for oncological response, in contrast to liver metastasis [661-668]. One hypothesis for this discrepancy is that T cells, the main effectors of immune checkpoint inhibitor activity, play a less significant role in normal tissue homeostasis, pre-metastatic niche formation, and metastatic growth in these organs compared to their role in the liver.

Therefore, their preservation in non-liver organs would support the maintenance of immune checkpoint inhibitor efficacy, as evidenced by higher objective response rate in patients with lymph node metastases [663, 664, 668]. Indeed, lymph nodes are the sites in which the antitumoral response takes place. Lymph nodes are continuously stimulated by tumoral neoantigens, which are processed by dendritic cells to induce CD4<sup>+</sup> and CD8<sup>+</sup> antitumoral responses favourable to immune checkpoint inhibitor treatment [688-690]. However, this situation creates a state of local chronic inflammation that favours the recruitment of immunosuppressive cells, such as Myeloid-derived suppressor cells, tumour-associated macrophages, and immature dendritic cells. In turn, this environment is favourable for the implantation and proliferation of cancer cells. This condition is further exacerbated by immunosuppressive signals that are released from the primary tumour, particularly interleukin-10 and TGF- $\beta$  (Figure A1.5) [689, 691, 692].

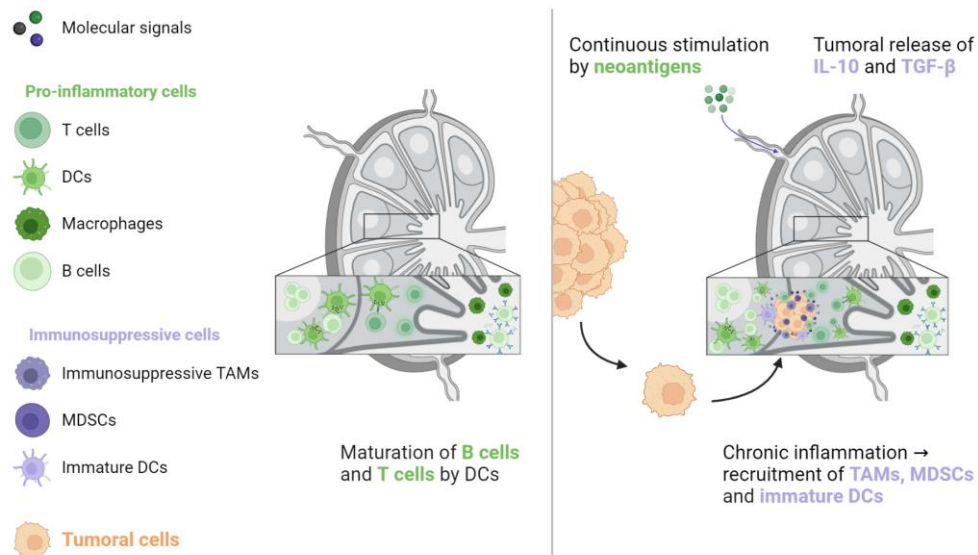


Figure A1.5: The activation of naive B and T lymphocytes takes place in lymph nodes. When these lymphocytes are continuously triggered by neoantigens from the primary tumour, antitumoral immunity is counterbalanced by immunosuppressive cells, including tumour-associated macrophages, Myeloid-derived suppressor cells, and immature dendritic cells. In addition, the primary tumour generates immunosuppressive signals that promote tumoral cell implantation and proliferation. DC: Dendritic cell, MDSC: Myeloid-derived suppressive cell, TAM: Tumour-associated macrophage, TGF- $\beta$ : Transforming growth factor- $\beta$ .

### 7.1.2.3. Heterogeneity

In addition to metastatic location, tumour immune microenvironment heterogeneity is another factor that may affect treatment outcomes, especially in the era of immune checkpoint inhibitors [538, 693-697]. In several malignancies, including non-small cell lung cancer, melanoma, renal cell carcinoma, and CRC, there is widespread acceptance of the existence of

a gene-expression heterogeneity between primary tumours and their metastases, which may impact treatment results [693, 698-700]. In many cancers, the tumour immune microenvironment in metastases is more immunosuppressive than that of the primary tumours [628, 699, 701-709].

Metastases located in the same organ share similar tumour immune microenvironment characteristics, regardless of the primary tumour. García-Mulero and colleagues reported the gene signature of 374 metastases located in four organs (lung, liver, brain, and bone) of various primary tumours. The tumour immune microenvironment of lung metastases was the most pro-immunogenic, with a high score for effector cells and a low score for suppressor cells, followed by bone, brain, and liver metastases [710]. The organ affected by metastases may not account for all sources of tumour immune microenvironment heterogeneity, but it may partly explain some tumour immune microenvironment variations. A study by Angelova and colleagues assessed spatial and temporal tumour immune microenvironment heterogeneities in two metastatic CRC patients with a long disease course, due to the availability of multiple tumour samples. Within the same patient, the tumour immune microenvironment differed between metastases and between different areas from the same lesion. Additionally, the tumour immune microenvironment may vary considerably between metastases that have a parent-child relationship, based on their similar mutational profiles. Globally, the rate of pro-immunogenic mutations seems to decrease in late-onset lesions, which favours the development of immune escape mechanisms and promotes disease progression. However, high immunoscore values, which reflect a high immune infiltration, can also



be found in tumour samples collected in later onset of metastatic diseases [711]. Similar tumour immune microenvironment heterogeneity has also been reported for metastases arising from the same organ in melanoma and ovarian cancer [712, 713].

Tumour immune microenvironment-based classification systems have been created to account for the effect of tumour immune microenvironment heterogeneity on patient prognosis. These systems may be more predictive of patient outcome than the American Joint Committee on Cancer/UICC Tumor-Node-Metastasis (TNM) classification, as is the “immunoscore” in CRC, for instance [75, 80, 83, 84].

### **7.1.3. Immune Priming Effect of Radiotherapy**

Dying irradiated tumour cells release tumoral neoantigens and damage-associated molecular patterns, which enhance dendritic cell maturation. In tumour-draining lymph nodes, activated dendritic cells present these neoantigens to naive T cells, which leads to the activation and clonal expansion of antitumoral T cells, which are then released into the systemic circulation. These activated T cells migrate and infiltrate the microenvironment of lesions distant from the irradiated site, where they can induce a tumoral immunogenic cell death [203-205]. This description is a simplified model of the RT-mediated immunological response, which involves additional immune cells—such as Tregs, Myeloid-derived suppressor cells, tumour-associated macrophages, natural killer cells, and dendritic cells—as well as endothelial cells and stromal cells. The “abscopal

effect” (i.e., the immunological response outside the RT field) was first described in 1953, and interest in this effect has increased with the emergence of immune checkpoint inhibitors treatment because the abscopal effect may improve the systemic efficacy of immune checkpoint inhibitors in insensitive tumours [647, 714]. Encouraging preclinical results have been recorded, but a deeper understanding of the RT-induced immunological response is needed to increase the frequency of occurrence of the abscopal effect, which remains scarce in humans [535, 648, 715].

#### *7.1.3.1. Local RT Effect*

RT can locally modify the tumour immune microenvironment, with variations dependent on the RT dose (Figure A1.6). Globally, regardless of dose level, increases have been observed in local infiltration by CD8<sup>+</sup> T and natural killer cells [220-224]. With low-dose RT (2 Gy per fraction), the tumour-associated macrophages polarisation changes to a more antitumoral M1 phenotype, whereas with higher dose of RT (>8 Gy per fraction), the induced tumour-associated macrophages M2 polarisation promotes tumour progression through angiogenesis stimulation [213, 226-228]. The beneficial effects of RT on dendritic cells also vary by dose level. At moderate (8-12 Gy per fraction) and high (>12 Gy per fraction) doses, RT increases the expression of MHC-II molecules and other co-stimulatory receptors for T cell priming. At low doses, it increases the production of pro-immunogenic cytokines such as interleukin-2, interleukin-12, and IFN- $\gamma$ , which promote T cell proliferation [221, 229, 230]. High-dose RT reduces the

number of infiltrating Myeloid-derived suppressor cells, whereas moderate- and low-dose RT enhance this number, creating an immunosuppressive environment [205, 231]. Tregs are considered radioresistant compared to other immune cells. In a murine model of prostate cancer, the proportion of Treg infiltration in tumour immune microenvironment increases linearly from 2 Gy to 20 Gy [225]. However, another preclinical study found that low-dose RT reversed the Treg tumoral infiltration mediated by earlier high-dose RT [226]. Finally, the effects of RT on the vasculature can also impact the metastatic tumour immune microenvironment. Immune cell migration is facilitated by low-dose RT because the treatment improves blood vessel permeability [210, 213, 214]. On the other hand, high-dose RT may lead to major blood vessel damage and restrict tumoral infiltration by immune cells [210, 628].

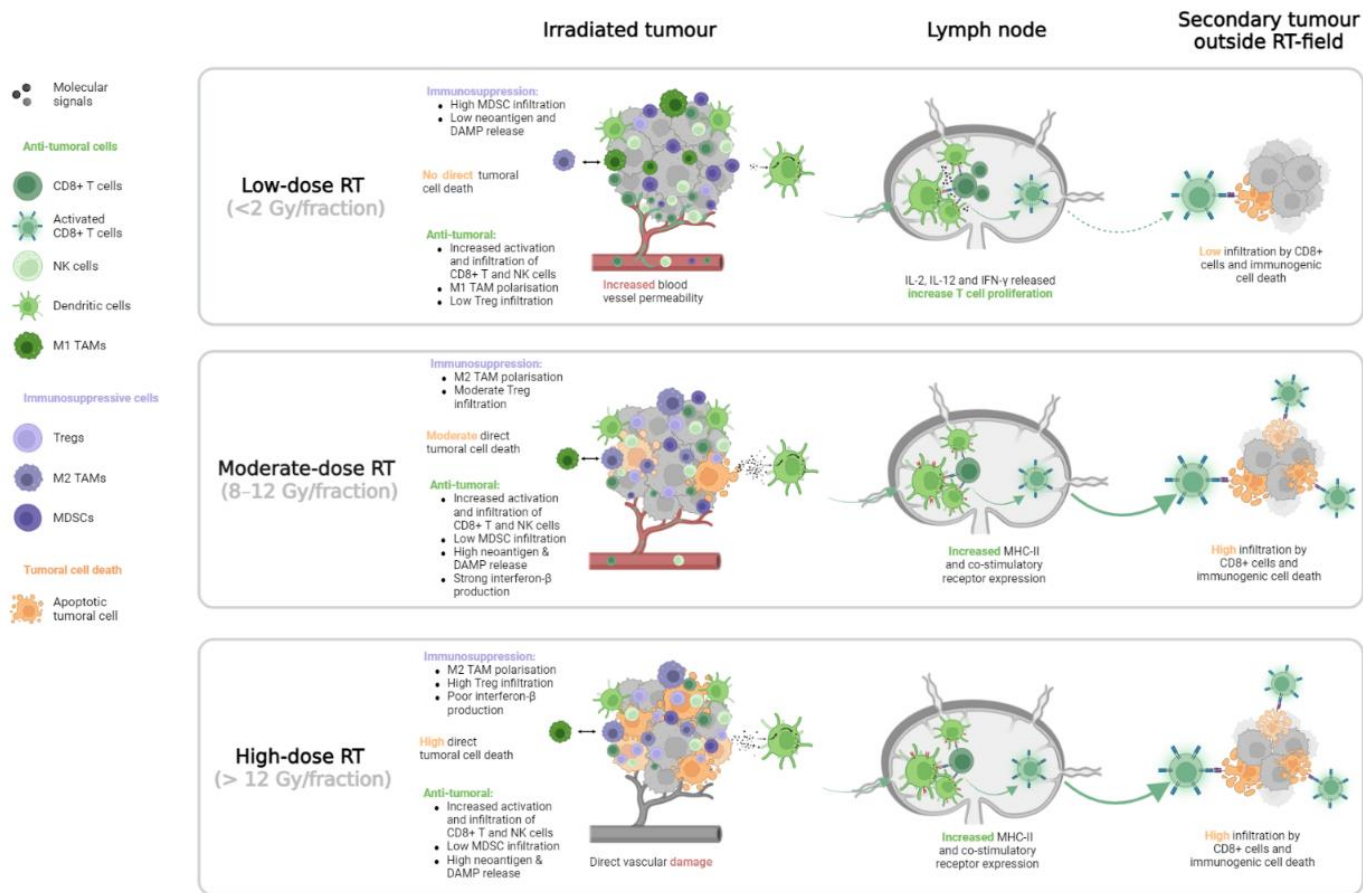


*Figure A1.6: Summary of modifications to the tumour immune microenvironment and systemic reactions induced by different RT dose levels. Unlike moderate- or high-dose RT, low-dose RT does not induce enough direct cell death to sufficiently release neoantigens and damage-associated molecular patterns, resulting in a weak distant immunological response. In the case of high-dose RT, induction of the Trex1 exonuclease decreases interferon- $\beta$  production, which is required for a strong abscopal response. Compared to high-dose RT, low-dose RT may reduce Treg infiltration in the irradiated tumour and promote M1-polarisation of tumour-associated macrophages, thereby inducing an antitumoral response. The effect of RT on Myeloid-derived suppressor cells is also dose dependent: moderate and high-dose RT decreases the infiltration of these immunosuppressive cells, whereas low-dose RT increases infiltration. Dendritic cells play a key role in CD8+ T cell priming in tumour-draining lymph nodes. At low-dose RT, this phenomenon is mostly due to dendritic cells' production of interleukin-2, interleukin-12, and IFN- $\gamma$ , but at higher doses, it occurs due to dendritic cells' enhanced expression of the MHC-II receptor. Finally, the reshaping of the tumour immune microenvironment is also dependent on the effect of RT on blood vessels. Low-dose RT increases vasculature permeability, which allows immune cells to migrate to the tumoral site. In the case of high-dose RT, immune cell migration is restricted due to direct RT-induced vasculature damage. DAMP: damage-associated molecular pattern, MDSC: Myeloid-derived suppressive cell, MHC: Major histocompatibility complex, NK: Natural killer, RT: Radiotherapy, TAM: Tumour-associated macrophage, Treg: Regulator T cell.*

#### 7.1.3.2. *Systemic Effect*

As for local responses, RT-mediated systemic immune reactions may vary with RT dose. In a preclinical study, Vanpouille-Box and colleagues discovered that high-dose RT (20 Gy per fraction) activates Trex1 exonucleases, which degrade the cytosolic DNA required for interferon- $\beta$ -mediated CD8<sup>+</sup> cell activation. This activation limits the abscopal response compared to that of moderate-dose RT (8 Gy per fraction) [233]. According to the findings of another preclinical study by Dewan and colleagues, fractionation is another component that may elicit the abscopal effect [233, 234]. Mice that received three doses of 8 Gy had a stronger induction of interferon- $\beta$  than mice that received a single dose of 8 Gy. However, low-dose RT (<2 Gy per fraction) did not induce sufficient direct tumoral cell death to release neoantigens and damage-associated molecular patterns and further increase CD8<sup>+</sup> T cell infiltration in distant tumours (Figure A1.6), compared to higher doses (>8 Gy) [235].

It is well established that RT can induce lymphopenia that can be long lasting and associated with poor clinical outcomes in several malignancies, whether used alone or in combination with chemotherapy or hormonal therapy [237, 716-718]. The severity of lymphopenia depends on the RT site and size of the RT fields. Absolute lymphocyte count (ALC) drop may be predicted by the RT dose delivered to haematopoietic organs, such as bone marrow, the spleen, or circulatory system [241, 719, 720]. In stereotactic ablative RT (SABR), the RT fields are reduced compared to conventional RT, which may limit the severity of lymphopenia. RT-mediated modifications of circulating

T cell populations also appear to be linked to the immune environment of the organ hosting the target lesion. In a Phase I study of 35 patients with various primary tumours, SABR was given to patients with lung metastases or liver metastasis in combination with ipilimumab. The patients treated with lung SABR had a significantly lower proportion of circulating CD8+ cells expressing inducible co-stimulatory molecule, glucocorticoid-induced TNFR-related protein (GITR), and lymphocyte-activation gene 3 (LAG-3) than patients treated with liver SABR. In addition, 4-1BB, GITR, T cell immunoglobulin, mucin containing protein-3 (TIM-3), and PD-1 were less expressed on circulating Tregs in patients treated with lung SABR compared to patients treated with liver SABR. Some of these markers are immunosuppressive (LAG-3, TIM-3, and PD-1), while others are pro-immunogenic (inducible co-stimulatory molecule, GITR, and 4-1BB). These data do not support the theory that liver RT induces a more immunosuppressive or pro-immunogenic response than lung RT. However, the researchers suggest that this difference reflects an earlier activation of the immune system when the liver is irradiated than when the lung is irradiated [721].

#### *7.1.3.3. Implications for RT-immune checkpoint inhibitors Combination*

The selection of the site to irradiate may play an important role in promoting the abscopal response. It may also induce objective responses in patients who do not respond initially to immune checkpoint inhibitors, such as those

with liver metastasis. Limited clinical data have been reported regarding the impact of the irradiated organ on the tumour immune microenvironment modifications and the abscopal effect occurrence. Only indirect indications can be extracted from immunoradiation trials. However, in most cases, these studies evaluated a small number of patients, and no strong evidence can be drawn (Table A1.1) [715, 722-731].



| Author                          | Primary                    | Immune checkpoint inhibitor | N RT Fractions | Dose Per RT Fraction (Gy) | Total Population |                       | Irradiation of lymph node Metastasis |                       | Irradiation of Lung Metastasis |                       | Irradiation of Liver Metastasis |                       |
|---------------------------------|----------------------------|-----------------------------|----------------|---------------------------|------------------|-----------------------|--------------------------------------|-----------------------|--------------------------------|-----------------------|---------------------------------|-----------------------|
|                                 |                            |                             |                |                           | N                | Abscopal Response (%) | N                                    | Abscopal Response (%) | N                              | Abscopal Response (%) | N                               | Abscopal Response (%) |
| Sundahl, 2019 [715]             | Melanoma                   | Nivolumab                   | 3              | 8                         | 20               | 9 (45.0)              | 7                                    | 5 (71.4)              | 5                              | 1 (20.0)              | 1                               | 0 (0)                 |
| Twyman-Saint Victor, 2015 [722] | Melanoma                   | Ipilimumab                  | 2 or 3         | 6 or 8                    | 20               | 4 (20.0)              | 2                                    | 0 (0)                 | 9                              | 1 (11.1)              | 3                               | 1 (33.3)              |
| Hiniker, 2016 [723]             | Melanoma                   | Ipilimumab                  | 1 – 15         | 2.5 – 25                  | 20               | 6 (30.0)              | 3                                    | 0 (0)                 | 6                              | 3 (50.0)              | 2                               | 0 (0)                 |
| Sundahl, 2018 [724]             | Melanoma                   | Ipilimumab                  | 3              | 8 – 12                    | 13               | 1 (7.7)               | 3                                    | 0 (0)                 | 3                              | 0 (0)                 | 1                               | 0 (0)                 |
| Maity, 2021 [725]               | Melanoma                   | Ipilimumab                  | 2 – 3          | 6 – 8                     | 22               | 5 (22.7)              | 6                                    | 1 (16.7)              | 10                             | 2 (20.0)              | 3                               | 1 (33.3)              |
| Formenti, 2018 [726]            | non-small cell lung cancer | Ipilimumab                  | 3              | 6 or 9.5                  | 21               | 7 (33.3)              | 1                                    | 0 (0)                 | 13                             | 5 (38.5)              | 3                               | 0 (0)                 |
| Qin, 2020 [727]                 | non-small cell lung cancer | Atezolizumab                | 2 – 5          | 6 – 10                    | 12               | 3 (25.0)              | 4                                    | 2 (50.0)              | 2                              | 1 (50.0)              | 1                               | 0 (0)                 |
| Sundahl, 2019 [728]             | Bladder                    | Pembrolizumab               | 3              | 8                         | 18               | 4 (22.2)              | 8                                    | 2 (25.0)              | 8                              | 2 (25.0)              | -                               | -                     |
| Voorwek, 2019 [729]             | TNBC                       | Nivolumab                   | 3              | 8                         | 12               | 1 (8.3)               | 5                                    | 1 (20.0)              | 1                              | 0 (0)                 | 1                               | 0 (0)                 |

|                   |         |                         |         |           |    |          |    |          |          |          |         |          |
|-------------------|---------|-------------------------|---------|-----------|----|----------|----|----------|----------|----------|---------|----------|
| Welsh, 2019 [730] | Various | Ipilimumab              | 4 or 10 | 6 or 12.5 | 95 | 9 (9.5)  | -  | 52       | 6 (11.5) | 43       | 3 (7.0) |          |
| Kong, 2022 [731]  | Various | PD-1 inhibitor & GM-CSF | 3       | 5 or 8    | 48 | 9 (18.6) | 16 | 3 (18.6) | 7        | 3 (42.9) | 7       | 2 (28.6) |

*Table A1.1: Summary of prospective trials evaluating abscopal response in patients treated with RT-immune checkpoint inhibitor combinations. Only studies reporting abscopal response occurrence according to the RT site were included. Abscopal response was defined as the occurrence of a complete or partial response as the best response observed in metastases outside the RT field, according to response evaluation criteria in solid tumours (RECIST) v1.1. GM-CSF: Granulocyte-macrophage colony-stimulating factor, non-small cell lung cancer: Non-small cell lung cancer, PD-1: Programmed cell death, RT: Radiotherapy, TNBC: Triple-negative breast cancer.*

Liver metastasis irradiation can reshape the tumour immune microenvironment and reverse systemic liver metastasis-induced immunosuppression. In mice, liver RT has been found to increase CD8<sup>+</sup> T cells and reduce Myeloid-derived suppressor cell infiltration in the tumour immune microenvironment. The addition of anti-PD-L1 immune checkpoint inhibitor amplifies this effect by enhancing specific populations of antitumoral CD8<sup>+</sup> cells that express Ki67, IFN- $\gamma$ , and granzyme B. Compared to the use of RT or anti-PD-L1 alone, the combination of these therapies increases CD8<sup>+</sup> T cell infiltration in non-irradiated tumours and reverses the systemic immune checkpoint inhibitor resistance induced by liver metastasis [538]. However, data regarding tumour immune microenvironment modifications induced by liver RT in humans remains limited. A combination of different RT dose levels with immune checkpoint inhibitors could also be effective at reversing the immunosuppressive state of liver metastasis, as was demonstrated in a preliminary post hoc analysis of a Phase II study. This study evaluated the combination of high- and low-dose RT with continuing immune checkpoint inhibitors beyond progression in non-small cell lung cancer and melanoma patients. The lesion-specific response rate after low-dose RT was higher for liver metastasis (71%, n = 7) than for lung metastases (29%, n = 17). In addition, T- and natural killer cell infiltration were enhanced in lesions treated with low-dose RT [262, 732].

Irradiation of tumour-draining lymph nodes is recommended as part of curative treatment for several cancers. However, since the immune response takes place in the tumour-draining lymph nodes, RT in these areas may be detrimental when using immune checkpoint inhibitors [247, 248].

The abscopal effect was reduced among mice that were irradiated at the tumoral site and the tumour-draining lymph nodes compared to those irradiated at the tumoral site only. The irradiation of tumour-draining lymph nodes decreases the stem-like CD8<sup>+</sup> T cells within the irradiated tumour, in distant tumour sites, and in their respective draining lymph nodes [247]. This observation occurred regardless of whether RT was combined with immune checkpoint inhibitors. Overall survival was lower in mice irradiated at the tumour site and tumour-draining lymph nodes compared to those only irradiated at the primary tumour, whether immune checkpoint inhibitors were directed against PD-1 or CTLA-4 [250]. Moreover, avoiding large volumes and haematopoietic organ irradiation enables reduction of RT-induced lymphopenia, which is associated with a lower overall survival in patients treated with an RT-immune checkpoint inhibitor combination [733]. This finding suggests that a preserved systemic immune system is required to maximise the results of the RT-immune checkpoint inhibitor combination. Thus, stereotactic treatments that reduce the total irradiated volume should be preferable in immunoradiation trials that aim to increase the occurrence of abscopal effect.

There is growing agreement that multisite RT, rather than single-site RT, should be used in combination with immune checkpoint inhibitors [257-260]. In a preclinical pancreatic tumour model, the response in a non-irradiated tumour was stronger and faster in mice with two irradiated sites than in those with only one [261]. Due to the molecular heterogeneity across metastases, targeting several lesions enhances the variety of tumoral neoantigens generated. The processing of these neoantigens by dendritic

cells increases the T cells' repertoire, which may result in a less stereotypical antitumoral response [258, 259]. However, when the metastatic burden is important, SABR may not be safely delivered to all sites without jeopardising the protection of the surrounding healthy tissue. In this case, a combination of moderate-dose (6 – 12 Gy) or high-dose (>12 Gy) per fraction RT on a single target and low-dose RT (total dose of  $\leq 10$  Gy in fractions  $\leq 2$  Gy) on all other tumour sites could be an option to promote tumoral infiltration by cytotoxic T cells and subsequently induce significant regression in lesions receiving low-dose RT. This phenomenon, called the "RadScopal effect", has been observed in several preclinical trials [258, 263, 734]. Patel and colleagues proposed a decision-making process to select the site and dose of RT based on the patient tumour burden and immune function. They suggested giving an ablative treatment with high-dose SABR to all sites in oligometastatic patients to improve both local control and the immune priming effect. Combining ablative RT on only one lesion with low-dose RT on every other lesion (or even the entire organ) in patients with high-volume disease may effectively stimulate the immune system without causing major toxicity. However, for immunocompromised patients, they expected only a poor immune reaction to be induced by low-dose RT. Therefore, they recommend radical doses for these patients [258]. In addition, having ALC before RT could potentially guide the radiation oncologist to making such a decision, since high ALC is predictive of abscopal response and progression-free survival in RT-immune checkpoint inhibitor combination [239, 258].

#### **7.1.4. Conclusions**

Since the development of immune checkpoint inhibitors, the characterisation of the tumour immune microenvironment has become an essential part of metastatic patient care. The tumour immune microenvironment can vary depending on the metastatic location and is shaped by the involved organ. Further, the metastatic location impacts the systemic anti-tumour immune effects since patients with liver metastases exhibit a poor response to immune checkpoint inhibitors compared with patients with metastases in other organs. Combination with RT is currently being investigated to improve immune checkpoint inhibitor response, given the immune priming effect induced by radiation. RT-mediated immune reaction is not only local but also systemic and can lead to responses in non-irradiated tumour sites in rare cases, which is known as the abscopal effect. Future RT-immune checkpoint inhibitor combinations must consider several technical aspects of the RT, such as dose, fractionation, and RT site, to increase the occurrence of such distant responses. These aspects require further investigation to guide radiation oncologists in optimising RT-immune checkpoint inhibitor combinations. Multisite RT should also be considered, if safe, to increase the diversity of the anti-tumour response.

## **7.2. Appendix 2: Cone-beam computed tomography-guided online-adaptive radiotherapy for inoperable right colon cancer: first in human**

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## **Abstract**

We report the case of a medically inoperable patient with localised colon cancer. Due to symptomatic bleeding, definitive radiotherapy (5 daily fractions of 5 Gy) has been performed using cone-beam computed tomography-based online-adaptive radiotherapy (oART). oART enables compensation of interfraction motion of abdominal organs by performing daily delineation of organs at risk (OARs) and target volumes. Daily treatment replanning maximised target volume coverage while lowering the dose to OARs. Intrafraction variation of the tumour was still significant and had to be incorporated in the planning target volume margin computation. After the treatment, the patient did not develop any acute radiotherapy-induced adverse events and had no further rectal bleeding either at the end of the radiotherapy or at oncological follow-up 4 months later. oART for colon cancer is feasible and is a valuable alternative when surgery is not an option.



### **7.2.1. Introduction**

The recommended treatment for localised colon cancer is surgery [7]. However, it may be contraindicated in patients with high comorbidity. Radiotherapy (RT) is not currently part of the management of localised colon cancer due to the lack of clinical benefit and significant toxicity [7, 735]. Hence, it is only considered a palliative haemostatic treatment in cases of tumoral bleeding [736]. Nevertheless, RT could improve the resection rate and the overall survival in selected patients, according to recent retrospective data [737, 738]. Colon RT is challenging due to poor tumour visualisation on cone-beam computed tomography (CBCT) and the high inter- and intra-fraction motion of the tumour and abdominal organs, including the colon itself [735, 738-741]. Online-adaptive radiotherapy (ART) offers an opportunity to overcome the challenges of colon RT as it facilitates daily replanning, compensating for anatomical interfraction variations.

### **7.2.2. Case Report**

#### ***7.2.2.1. Clinical data***

A 71-year-old man presented with rectal bleeding and melena over the previous 15 days. His medical history is characterised by an oxygen-dependent chronic obstructive pulmonary disease (COPD GOLD IV), atrial fibrillation, for which he receives anticoagulant therapy, and obesity (body mass index = 30.5 kg/m<sup>2</sup>). Blood tests revealed a grade 1 microcytic anaemia (Hb = 11.5 g/dL). A colonoscopy showed a tumour in the caecum. The contrast-enhanced thoraco-abdominal CT did not detect any distant lesions.

Biopsies confirmed the diagnosis of microsatellite-stable adenocarcinoma, staged cT2N0M0 (tumour, node, metastasis classification, 8th edition).

The patient was deemed inoperable and unfit for chemotherapy due to excessive comorbidities. Simple follow-up was proposed. During the following weeks, the ongoing rectal bleeding led to grade 3 anaemia (Hb = 7.9 g/dL), with a deterioration of the pre-existing grade 4 dyspnea, which required blood transfusions. RT treatment was suggested despite the lack of recommendations, aiming for haemostatic and local tumour control. This motivated us to develop an online-adaptive RT (oART) protocol tailored to these specific conditions using our o-ring gantry linear accelerator (ETHOS; Varian Medical Systems, Palo Alto, Calif., USA).

#### *7.2.2.2. oART technique*

Based on the short-course neoadjuvant RT scheme recommended for rectal cancer, we prescribed a dose of 25 Gy in five consecutive daily fractions to the planning target volume (PTV) [285].

The planning-CT was acquired in fasting condition (nihil by mouth for one hour prior to planning-CT acquisition), with oral butylhyoscine premedication, and an empty bladder. The patient was positioned in supine position without any immobilisation device besides soft immobilisation cushions (Orfit®, Belgium), arms backwards. Abdominal compression was not used in order to avoid additional breathing difficulties for the patient.

Volume delineation was made on Raystation (clinical version 12A, RaySearch Laboratories, Stockholm, SE). The clinical target volume (CTV) consisted of the circumferential colonic wall on each of the transversal CT slices on which the tumour was apparent. An initial plan was created with the treatment planning system of the ETHOS (version 2.0).

During the oART procedure, the CTV of the day was generated using the OARs-guided deformable registration algorithm of the ETHOS between the planning-CT and the CBCT. The volume of the CTV was then manually edited in order to include the entire colon wall, extending from the top of the most cranial radiopaque markers to bottom of the most caudal one.

Three important technical aspects were considered for the planning and the oART process of such an unusual location: the interfraction variation, the intrafraction variation, and the lack of visibility of the tumour on CBCT for the image-guided RT procedure.

The interfraction variation of the colon is the most significant one, with reported values reaching up to 35 mm [739]. The on-board treatment planning system of the ETHOS allowing oART can compensate for those interfraction variations [190]. It carries out oART with daily automatic delineation of OARs, OARs-guided deformable propagation of target volumes, and daily replanning within an acceptable time (23.8 minutes for this case in average, Figure A2.1C). This way, oART suppresses interfraction variations and setup uncertainties.

oART does not address intrafraction anatomical variations. We therefore considered three actions to minimise them. (1) Twenty milligrams of oral

butylhyoscine were taken orally 30 minutes before each oART session to decrease bowel motility due to its antispasmodic action. (2) Three CBCTs were performed for repeated image-guided RT (“pelvis large fast” mode of the ETHOS – 25 seconds duration) during each oART session. The first CBCT was used for daily replanning. The second CBCT was made just before RT delivery to assess potential intrafraction displacement. At this point, no treatment was delivered yet and a subsequent table displacement or a new replanning could be made if required. The third CBCT, acquired after RT delivery, was used to evaluate if the final intrafraction displacements of the CTV did not extend outside the PTV. (3) Finally, an empirical isotropic PTV of 20 mm was added around the CTV. However, based on clinician medical decision, the PTV was reduced in the right direction down to 10 mm due to the abutment of the tumour with the abdominal wall (Figure A2.1B).

Finally, to address the poor visibility of the tumour on CBCT, six radio-opaque markers (vascular clips) were implanted around the circumference of the tumour, including two at its upper and lower poles. This was performed during a colonoscopy, one day before the RT planning (Figure A2.1A). These markers were used as surrogates for tumour position during the whole treatment.

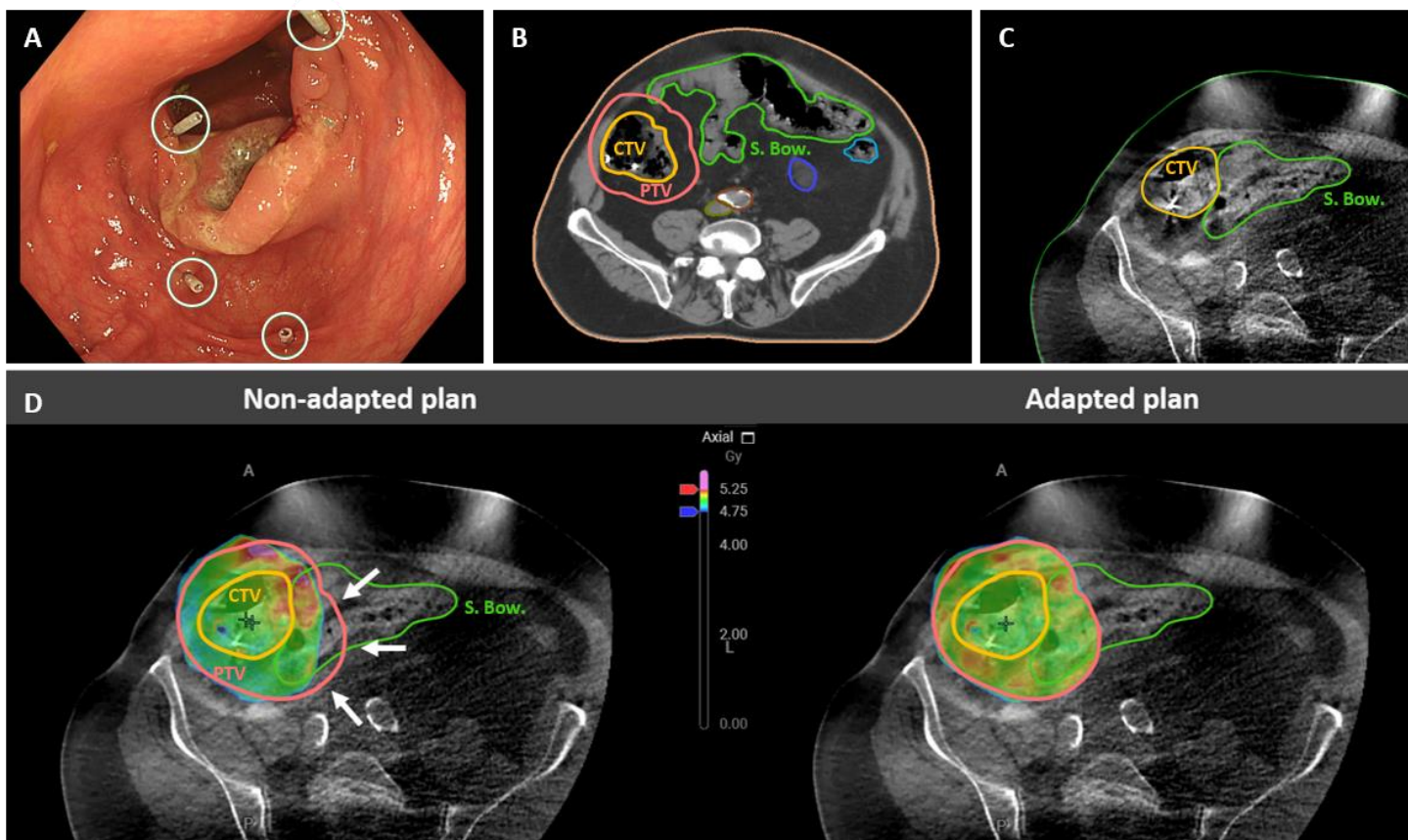


*Figure A2.1: (A) Six radio-opaque markers were inserted endoscopically in the normal colon mucosa around the tumour the day before the radiotherapy planning CT. (B) These markers were used to delineate the CTV on the planning CT. It is defined as the colonic wall from the CT-slice above the first visible marker to the CT-slice below the last marker in a craniocaudal direction along the colonic axis. PTV consisted of an isotropic margin of 20 mm in all directions except 10 mm in the right direction around CTV. (C) CTV position was propagated on the cone-beam CT of the day using the influencer-guided deformable image registration algorithm for each oART session. The small bowel (S. Bow.) was one of the organs at risk considered for this treatment. It was automatically delineated by the artificial intelligence-based algorithm. Manual corrections were done for both the CTV and organs at risk if required. (D) A new dosimetry was computed each day by the oART treatment planning system. This allowed the radiation oncologist to choose the best plan between non-adapted and adapted plans. Adapted plans allow for better coverage of the PTV compared to the non-adapted plan (white arrows). ART: Adaptive radiotherapy, CT: Computed tomography, CTV: Clinical target volume, PTV: Planning target volume.*

### 7.2.2.3. *Treatment assessment*

We report here below the treatment time requested for all treatment sessions, regarding each step of the adaptive workflow. We also report the adaptive results of each sessions regarding dose to OARs and target volume coverage.

An additional retrospective analysis was performed in order to assess both intra- and inter-fraction variations based on the CTV delineation reported on all the CBCTs acquired during the treatment. We used two metrics: the mean distance-to-agreement (mDTA), which evaluates the average distance between structures, and the 95% Hausdorff distance (HD95), which is a surrogate for the largest distance between structures. For the interfraction variation, comparisons were made between the CTV delineated on the planning-CT and the CTV delineated on the first CBCT (CTV1) of each session after a rigid bony image registration. Additionally, we created a structure from the merging of all those CTV1, which was compared to the planning-CTV, to get an idea of the optimal margin that would be needed in a non-adaptive workflow to compensate for the interfraction variations. For the intrafraction evaluation, the same metrics were assessed by comparing CTV1 and the union of the CTV volumes delineated on all the CBCTs from the same session. Intrafraction tumoral motion was also quantified by analysing the displacement of radio-opaque markers during each session. We delineated a volume including all the radio-opaque markers on the three daily CBCTs and reported the distance between the centres of mass ( $\Delta\text{CoM}$ ) of these structures as a function of time from the first CBCT.

Finally, patient's tolerance to the treatment was also assessed by a physician following each RT sessions. The evolution of the initial rectal bleeding and the RT-induced adverse events were reported using the Common Terminology Criteria for Adverse Events (CTCAE) v5.0. at 4 timepoints: at treatment initiation, at treatment completion, 2 weeks after RT completion, and finally at 4 months post RT.

#### *7.2.2.4. Treatment results*

Figure A2.2 depicts the workflow of one oART session.

The oART procedure lasted on average 23.8 minutes (range: 20.6-26.9 minutes), from the start of CBCT acquisition to the end of the RT delivery. Most of this time was allocated to the online edition of OARs and CTV (average: 8.9 minutes, Figure A2.2). For the CTV daily delineation, we modified the volume generated by the ETHOS as proposed in our methodology, extending from the top of the most cranial radiopaque markers to bottom of the most caudal one. The tumour had the same tissue density than the surrounding colon wall, therefore, making its precise visualization impossible on the CBCT (Figure A2.1C).



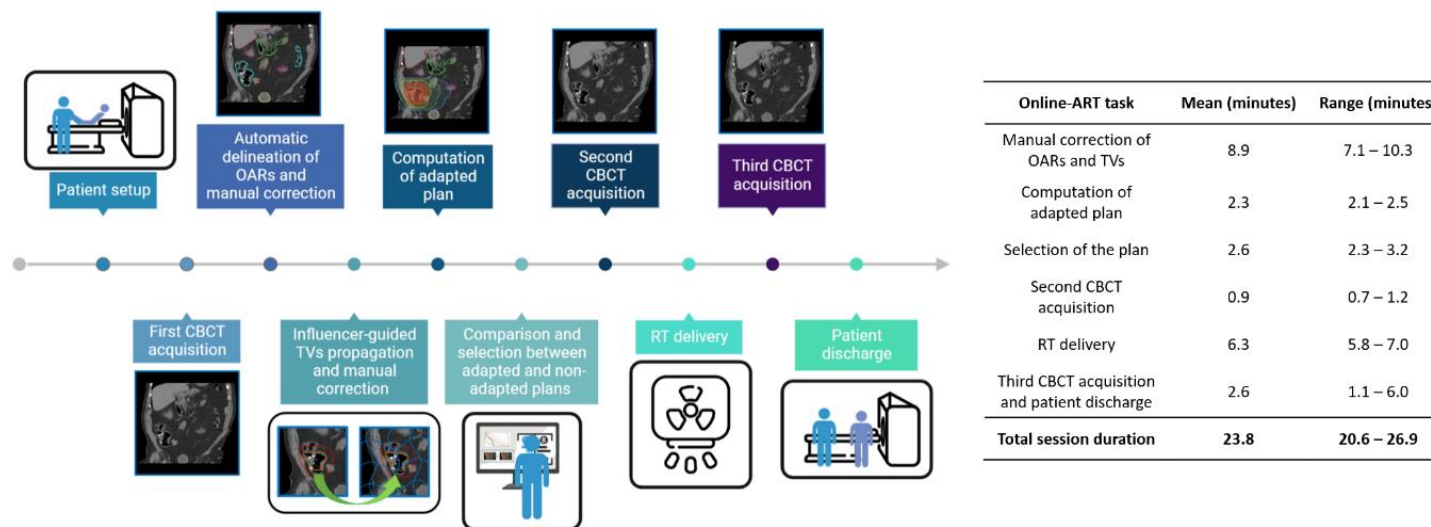


Figure A2.2: Workflow of a CBCT-guided oART session with duration of the different steps. ART: Adaptive radiotherapy, CBCT: Cone-beam computed tomography, OARs: Organs at risk, RT: Radiotherapy, TVs: Target volumes.

For all sessions, the radiation oncologist chose the daily adapted plan instead of the non-adapted plan. When comparing dosimetric data between both plans for each session, OARs sparing and target volumes coverage were better with the adapted plan (Figure A2.1D, Table A2.1).

|                                  | Non-adapted<br>(mean +/- SD) | Adapted<br>(mean +/- SD) | $\Delta$ |
|----------------------------------|------------------------------|--------------------------|----------|
| <b>Organs at risk constraint</b> |                              |                          |          |
| Small bowel D0.1 cc              | 5.6 +/- 0.1 Gy               | 5.3 +/- 0 Gy             | - 0,3 Gy |
| Small bowel D5 cc                | 5.3 +/- 0.1 Gy               | 5.1 +/- 0 Gy             | - 0,2 Gy |
| Sigmoid colon D0.1 cc            | 5.3 +/- 0.1 Gy               | 5.2 +/- 0.1 Gy           | - 0,1 Gy |
| Sigmoid colon V5 Gy              | 5.8 +/- 2.5 cc               | 6.8 +/- 4.8 cc           | + 1,1 cc |
| Skin D0.1cc                      | 4.9 +/- 0.1 Gy               | 5.0 +/- 0.1 Gy           | + 0,1 Gy |
| <b>Target volumes coverage</b>   |                              |                          |          |
| PTV D95 %                        | 81.5 +/- 13.9 %              | 99.8 +/- 0.2 %           | + 18,4 % |
| CTV D98 %                        | 99.1 +/- 2.3 %               | 99.6 +/- 0.5 %           | + 0,5 %  |

*Table A2.1: Dosimetric comparison between the non-adapted and daily-adapted plans for each individual session. Organs at risk constraints for which values did not exceed 10% of the objective were not reported.  $\Delta$ : Difference, CTV: Clinical target volume, PTV: Planning target volume, SD: Standard deviation.*

For the interfraction motion, the average mDTA and HD95 between the planning-CTV and the CTV delineated on the first CBCT of each session (CTV1) were 6.1 +/- 2.5 mm and 14.7 +/- 4.6 mm respectively. The intrafraction motion was quantify by using the average mDTA and HD95 between the CTV1 and the union of the CTV delineated on all the CBCTs from the same session. These values reached 1.9 +/- 0.6 mm and 8.3 +/- 2.3 mm, respectively (Table A2.2). The interfraction motion was thus superior to the intrafraction motion. The average intrafraction displacement ( $\Delta\text{CoM}$ ) of the

markers was found to be 7.0 mm (range: 3.5 – 12.4 mm), mostly in the antero-posterior axis. Our PTV margin was sufficient to encompass all these intrafraction variations of the tumoral colon wall. At the time of the second CBCT acquisition, online evaluation of the intrafraction motion was made by the physician. No replanning was required due to intrafraction extend of the CTV outside the PTV at this time.

| Interfraction variation |               |               | Intrafraction variation |               |               |
|-------------------------|---------------|---------------|-------------------------|---------------|---------------|
|                         | CTV mDTA (mm) | CTV HD95 (mm) |                         | CTV mDTA (mm) | CTV HD95 (mm) |
| Session 1               | 5.7           | 12.8          | Session 1               | 2.5           | 9.9           |
| Session 2               | 4.2           | 13.7          | Session 2               | 2.5           | 10.0          |
| Session 3               | 9.2           | 18.2          | Session 3               | 1.9           | 7.0           |
| Session 4               | 8.2           | 20.3          | Session 4               | 1.1           | 4.9           |
| Session 5               | 3.3           | 8.6           | Session 5               | 1.7           | 9.6           |
| Total CTV               | 6.5           | 17.0          | Mean +/- SD             | 1.9 +/- 0.6   | 8.3 +/- 2.3   |

*Table A2.2: For interfraction assessment, mDTA and HD95 were measured between the planning-CTV and the CTV delineated on the first CBCT of the oART session. The total CTV value was obtained by comparing the planning-CTV and the union volume of all CTV delineated on each first CBCT of all sessions. For intrafraction variation measurement, the same metrics were reported between the CTV delineated on the first CBCT of the oART session and the union of the CTV delineated on each of the three CBCTs of the session. CBCT: Cone-beam computed tomography, CTV: Clinical target volume, HD95: 95% Hausdorff distance, mDTA: Mean distance-to-agreement, SD: Standard deviation.*

Regarding patient's tolerance to the treatment, the patient never complained about the treatment duration nor declared any discomfort.

At the RT initiation, a grade 2 rectal bleeding was reported by the patient. At the RT end, the rectal bleeding had stopped (grade 0), and no further RT-induced toxicity was reported. After treatment completion, the rectal bleeding never occurred again, and no RT-induced toxicity was reported.

Four months post RT, a colonoscopy showed a major tumour response (> 50%) but biopsies demonstrated the persistence of adenocarcinoma cells.

### **7.2.3. Discussion**

Although the majority of adaptive treatments happen for pelvic RT treatments due to large and stochastic variations in anatomy, ETHOS has been used for other tumour locations, such as pancreatic and lower oesophagus cancers [501, 742]. To our knowledge, this is the first case of oART treatment for a colon cancer. This case demonstrates that oART for colon tumours is feasible in clinical practice. Indeed, the patient experienced neither discomfort nor adverse events from the RT treatment and deemed the treatment duration acceptable. The time from CBCT reconstruction to plan acceptance (13.9 minutes on average) was mostly allocated to the manual correction of automatically delineated OARs and CTV during the oART procedure (8.9 minutes on average, Figure A2.2). This is consistent with reports by Schiff et al. who assessed the oART feasibility in upper-abdominal and pancreatic tumours [313, 314]. In their *in silico* studies, the

mean duration was 22.6 minutes for upper abdominal locations (11.4 minutes dedicated to OAR delineation) and 36.3 minutes (24.0 minutes dedicated to OAR delineation) for pancreatic tumours [313, 314].

Colon cancer RT is scarcely performed in clinical practice. In the intergroup protocol 0130, patients with locally advanced colon cancer who received RT and chemotherapy combination in an adjuvant setting had a 5-year overall survival probability of 58%, compared to 62% for the patients treated with chemotherapy alone ( $p > 0.50$ ) [735]. Furthermore, patients in the RT group had more grade  $\geq 3$  adverse events ( $p = 0.04$ ). However, as in other studies evaluating RT in colon cancer, the intergroup protocol 0130 did not use modern RT techniques such as intensity-modulated radiation therapy or systematic CBCT-based image-guided RT, not even mentioning oART [735, 743].

By using daily delineation and planning to consider the patient's anatomical variations, oART could be a solution to deal with the interfraction deformation of the whole colonic wall (used to delineate the CTV) and the other abdominal organs such as the small bowel and sigmoid colon. However, it does not compensate for intrafraction variations, which remains the greatest source of uncertainty in oART. Also, colon RT must deal with the lack of visibility of the tumour and inter- and intra-fraction variations of the abdominal organs. Previous reports suggest the use of radio-opaque markers to guide image-guided RT [735, 741]. Magnetic resonance-guided RT could provide an attractive alternative given the direct visualisation of

the tumour it allows, making it possible to avoid radio-opaque markers, as is already the case for ART of rectal cancers [744].

Since our analysis is based on a single patient, further confirmations and longer follow-ups are needed in larger studies to determine whether oART for localised colon cancer could be an alternative when surgery is contraindicated in a radical setting. Although the tumour response is incomplete, a significant partial response and symptomatic control were achieved in this case. Based on these promising results, oART is an attractive technique for symptom palliation or tumour control and should be considered in future trials in colon cancer.

#### **7.2.4. Conclusion**

We present the first case of exclusive oART for a localised colon cancer. This technique was feasible and led to better target volume coverage and OAR sparing compared to a non-adaptive strategy. The unpredictability of the interfraction variation of the abdominal organs can be mitigated by oART. The insertion of radio-opaque markers is required to guide daily CTV delineation throughout the oART procedure due to the lack of visibility of the tumour on CBCT. There are still concerns about stochastic intrafraction variations. This should encourage larger-scale analyses to get a population-based quantification that may be integrated into PTV margin.

### **7.3. Appendix 3: Does prior radiation therapy impact the acute cellular liver graft rejection?**

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## **Abstract**

### *Purpose*

Radiotherapy can be used as a bridge therapy prior to liver transplantation. Radiotherapy generates immune reactions involving T cells, which are the main effectors of acute cellular rejection after transplantation. Here, we investigated the impact of radiotherapy on acute cellular rejection.

### *Materials and methods*

We retrospectively reviewed oncological patients who benefited from liver transplantation. Patients who received radiotherapy prior to liver transplantation (RT cohort, n=17) were compared to a matched cohort (NoRT<sub>matched</sub> cohort, n=17) obtained through propensity score matching analysis of the total non-irradiated cohort (NoRT<sub>all</sub> cohort, n=136). The acute cellular rejection was evaluated using the Banff score for rejection (mild: < 5, moderate: 5 – 6, and severe: 7 – 9) obtained on an early post-transplantation biopsy. Overall and disease-free survival were reported for hepatocellular carcinoma patients.

### *Results*

Median Banff scores was significantly lower for the RT cohort compared to the NoRT<sub>all</sub> cohort (2.5 versus 5, respectively, p=0.043) but this statistical difference was eliminated after comparison with the NoRT<sub>matched</sub> cohort (median: 4, p=0.62). The 5-year overall and disease-free survival were 62% and 69%, respectively, for hepatocellular carcinoma patients of the RT cohort (n=14) and did not differ from the 5-year overall (83%, p=0.15) and disease-free survival (90%, p=0.05) of those of the NoRT<sub>matched</sub> cohort (n=16).

### *Conclusion*

Radiotherapy given prior to liver transplantation did not impact the rate or severity of acute cellular rejection. Furthermore, overall and disease-free survival were not impacted by the radiotherapy.

### **7.3.1. Introduction**

Liver transplantation is the recommended first-line curative treatment for localised hepatocarcinoma and could be an option in early stage unresectable peri-hilar cholangiocarcinoma [480, 745]. However, the availability of grafts remains an important limitation for these patients and leads to a long time between diagnosis and transplantation. To control the tumour until liver transplantation, “bridge” treatments such as transarterial chemoembolisation or radiofrequency ablation could be indicated [480, 746, 747]. Liver stereotactic ablative radiotherapy (SABR) is another bridge strategy that has been recently demonstrated to be feasible with good local control and poor toxicities in localised hepatocarcinoma [514]. Conventional radiotherapy (RT) in combination with chemotherapy has been evaluated as neoadjuvant treatment before liver transplantation in selected cholangiocarcinoma patients according to the Mayo Clinic protocol [748]. Therefore, it is not uncommon for liver transplantation patients to have previously been irradiated. In addition to tumoral cell death induction, RT also mediates local and systemic immune reactions [25, 749]. Particularly, lymphopenia is a well-known effect of RT that can be long-lasting and is a poor prognosis factor for overall survival in several malignancies [237, 716, 750]. These RT-induced immune reactions could potentially impact the outcome of liver transplantation patients, who require immunosuppressive drugs to avoid liver graft rejection. Acute cellular rejection occurs in 15-25% after tacrolimus-based scheme. As T cells are the main mediators of acute cellular rejection, the RT effects on these cells could potentially impact acute cellular rejection frequency and severity [751]. This could have a detrimental

impact on oncological outcomes, since acute cellular rejection requires further immunosuppressive treatments such as steroids and other immunosuppressive drugs [752], and increased immunosuppression has been associated with a higher hepatocarcinoma recurrence rate after liver transplantation [753]. The aim of this retrospective propensity score-matching analysis is to evaluate if prior liver RT has a predictive value for acute cellular rejection.

### **7.3.2. Methods**

This research was conducted in accordance with the Strengthening the reporting of observational studies in epidemiology (STROBE) criteria for case-control studies [754].

#### **7.3.2.1. Patient selection**

Patients who underwent liver transplantation for an oncological indication were retrospectively included from January 2015, the date of liver RT implementation at our institution, until January 2023. Paediatric patients, patients with a history of prior non-liver RT, and patients with a history of prior liver transplantation before liver RT were excluded. The first cohort included liver transplantation patients with previous liver RT (RT cohort). For the comparative group, we used a propensity score-matching analysis to select patients sharing similar baseline characteristics with those from the RT cohort (NoRT<sub>matched</sub> cohort) among the whole cohort of liver

transplantation patients without RT history (NoRT<sub>all</sub> cohort). All patients meeting these criteria were included regardless of follow-up time. This monocentric study was conducted in accordance with the declarations of Helsinki and Istanbul and was approved by the ethics committee of Cliniques Universitaires Saint-Luc (reference number: 2023/17JUL/325). None of the subjects refused to consent to the publication of their data.

#### 7.3.2.2. *Procedures*

Included patients received initial tacrolimus after liver transplantation, as described in a previous study by our group [755]. We also included patients from a randomised protocol (EudraCT number 2006–004830–34) comparing initial tacrolimus monotherapy and combination of tacrolimus with a single intraoperative high-dose of rabbit anti-lymphocytic serum (r-ATG) [756]. The serum level of tacrolimus was regularly assessed, and a dose adaptation was performed to reach the recommended range of 4 to 6 ng/ml. A liver biopsy was routinely performed seven days after liver transplantation or earlier in cases of unexplained liver enzyme perturbation. Acute cellular rejection severity was classified by one experienced pathologist specialised in liver diseases (> 80 liver transplantations/year in our centre) using Banff criteria as mild (score < 5), moderate (5 – 6), and severe (7 – 9) [757]. In case of moderate or severe rejection, the tacrolimus dose was first adapted. If insufficient, further immunosuppressive drugs (steroids, mycophenolate mofetil, etc.) were added. Regular follow-up was performed to assess liver transplantation outcomes and oncological recurrence.

Patients with progression after alternative bridge treatment (transarterial chemoembolisation or radiofrequency ablation) as well as those with anatomical considerations that contraindicated invasive bridge therapy were referred to the RT department for liver SABR. Liver SABR was delivered using intensity-modulated radiation therapy. Prescribed doses ranged from 36 Gy to 50 Gy in three to six fractions (Biological effective dose range with an  $\alpha/\beta$  ratio = 10 : 57.6 – 124.8 Gy). For unresectable peri-hilar cholangiocarcinoma, neoadjuvant RT was performed in combination with 5-fluorouracil or capecitabine chemotherapy according to the Mayo Clinic protocol [748]. The liver hilum and associated lymph node regions received a prophylactic dose of 45 – 50 Gy in 25 - 30 fractions, and a 60 Gy simultaneous integrated boost to the gross tumour volume (GTV) was delivered in some cases. For all patients, we performed a planning 4D-computed tomography (CT) under audio-coaching and, if required to limit respiration motion, we applied abdominal compression. The GTV was delineated on the planning CT after its co-registration with a planning magnetic resonance imaging. An isotropic clinical target volume (CTV) margin of 5mm was added around the GTV for SABR treatment. The planning target volume margin was calculated according to the institutional standard Mid-position strategy [173]. Daily cone-beam CT was realised for the image-guided radiotherapy process.

#### *7.3.2.3. Endpoints*

To assess the impact of prior RT treatment on liver graft acute cellular rejection, anatomopathological reports from the first early liver biopsy were screened. A comparison was made between Banff scores of the RT cohort and the NoRT<sub>matched</sub> cohort. Survival endpoints were reported only for patients with hepatocarcinoma, since the oncological outcomes of cholangiocarcinomas are not similar. Time zero for overall survival and disease-free survival corresponds to the liver transplantation procedure date. Overall survival and disease-free survival were calculated until the date of the patient's death and the date of diagnosis of recurrent disease, respectively.

#### *7.3.2.4. Statistical analysis*

We performed a propensity score-matching to minimise the risk of imbalance between the RT cohort and the liver transplantation patients without RT. A logistic regression model considering covariables predictive of acute cellular rejection was used for the propensity score determination. Included covariables were: age, gender, body mass index (BMI), preoperative diabetes mellitus, autoimmune disease, pre-existing liver disease, tumour histology, Child-Pugh score class, Model for end-stage liver disease (MELD) score, cytomegalovirus status, warm and cold ischemia times, initial immunosuppressive therapy, blood levels of tacrolimus, and heart-beating/non-heart-beating donor status. Patients with missing data for these covariables were excluded from the propensity score-matching

analysis. A one-to-one nearest neighbour method without replacement was performed to find, among the NoRT<sub>all</sub> cohort, patients that had the closest propensity score. After matching, baseline characteristics were compared between the NoRT<sub>matched</sub> cohort and the RT cohort. The comparison of continuous variables (expressed as the mean +/- standard deviation or the median and interquartile range when appropriate) was made using the Wilcoxon signed-rank test. Chi-squared and Fisher's exact tests were performed to compare discrete variables when appropriate. Overall survival and disease-free survival were analysed according to the Kaplan–Meier method, and the log-rank test was used for comparison. A p-value < 0.05 was considered significant. All these statistical analyses were performed on RStudio (R version 4.2.1) using the “tidyverse”, “rstatix”, “survival” and “MatchIt” packages.

### **7.3.3. Results**

#### **7.3.3.1. Population**

During the evaluation period, a total of 160 patients underwent liver transplantation for an oncological indication. Of these, 7 patients were excluded due to missing data (n = 3), prior RT for another indication (n = 2), and second liver transplantation (n = 2). Among the 153 remaining patients, 17 were included in the RT cohort and 136 in the NoRT<sub>all</sub> cohort. Baseline characteristics were balanced for both cohorts except for BMI (p = 0.024) and tumour histology (p = 0.003). After matching, there was no further difference between the RT cohort and the NoRT<sub>matched</sub> cohort (Table A3.1).



| Characteristics               | RT cohort (n = 17) | NoRT <sub>all</sub> cohort (n = 136) |              | NoRT <sub>matched</sub> cohort (n = 17) |      |
|-------------------------------|--------------------|--------------------------------------|--------------|-----------------------------------------|------|
|                               |                    | p                                    |              | p                                       |      |
| Age (years)                   | 59.6 +/- 10.2      | 60.4 +/- 9.8                         | 0.67         | 62.0 +/- 8.4                            | 0.46 |
| Gender                        |                    |                                      |              |                                         |      |
| Male                          | 14                 | 111                                  | 0.99         | 13                                      | 0.99 |
| Female                        | 3                  | 25                                   |              | 4                                       |      |
| BMI (kg/m <sup>2</sup> )      | 30.5 +/- 5.6       | 27.5 +/- 5.2                         | <b>0.024</b> | 28.3 +/- 4.8                            | 0.23 |
| Preoperative DM               |                    |                                      |              |                                         |      |
| Absent                        | 9                  | 76                                   | 0.99         | 10                                      | 0.99 |
| Present                       | 8                  | 60                                   |              | 7                                       |      |
| Autoimmune disease            |                    |                                      |              |                                         |      |
| Absent                        | 16                 | 131                                  | 0.51         | 17                                      | 0.99 |
| Present                       | 1                  | 5                                    |              | 0                                       |      |
| Pre-existing liver disease    |                    |                                      |              |                                         |      |
| Alcoholic steatohepatitis     | 6                  | 59                                   | 0.23         | 7                                       | 0.78 |
| HCV                           | 3                  | 19                                   |              | 4                                       |      |
| Absent                        | 2                  | 19                                   |              | 1                                       |      |
| Multifactorial                | 1                  | 16                                   |              | 1                                       |      |
| Non-alcoholic steatohepatitis | 2                  | 10                                   |              | 2                                       |      |
| HBV                           | 1                  | 7                                    |              | 2                                       |      |
| Hemochromatosis               | 0                  | 3                                    |              | 0                                       |      |
| Other                         | 2                  | 3                                    |              | 0                                       |      |
| Tumour histology              |                    |                                      |              |                                         |      |
| hepatocarcinoma               | 14                 | 110                                  | <b>0.003</b> | 16                                      | 0.60 |
| Hepatocholangiocarcinoma      | 0                  | 7                                    |              | 0                                       |      |
| Cholangiocarcinoma            | 3                  | 1                                    |              | 1                                       |      |
| Colorectal metastases         | 0                  | 7                                    |              | 0                                       |      |
| NET metastases                | 0                  | 9                                    |              | 0                                       |      |
| Other                         | 0                  | 2                                    |              | 0                                       |      |
| Child-Pugh score class        |                    |                                      |              |                                         |      |

|                                          |             |                |      |              |      |
|------------------------------------------|-------------|----------------|------|--------------|------|
| Absence of cirrhosis                     | 1           | 20             |      | 1            |      |
| A                                        | 9           | 74             | 0.71 | 9            | 0.89 |
| B                                        | 5           | 31             |      | 5            |      |
| C                                        | 2           | 11             |      | 2            |      |
| <b>MELD score</b>                        | 12 [8 – 15] | 10 [7.75 – 15] | 0.42 | 12 [10 – 18] |      |
| <b>Cytomegalovirus status</b>            |             |                |      |              |      |
| Donor - / Recipient -                    | 2           | 32             |      | 3            |      |
| Donor + / Recipient -                    | 4           | 42             | 0.38 | 5            | 0.90 |
| Donor + / Recipient +                    | 6           | 40             |      | 3            |      |
| Donor - / Recipient +                    | 5           | 21             |      | 6            |      |
| <b>Cold ischemia time</b>                | 411 +/- 107 | 390 +/- 163*   | 0.51 | 420 +/- 169  | 0.85 |
| <b>Warm ischemia time</b>                | 41 +/- 11   | 46 +/- 14*     | 0.22 | 38 +/- 12    | 0.44 |
| <b>Initial immunosuppressive therapy</b> |             |                |      |              |      |
| Tacrolimus                               | 17          | 124            | 0.43 | 17           | 0.99 |
| Tacrolimus + r-ATG                       | 0           | 12             |      | 0            |      |
| <b>Tacrolimus blood level</b>            | 7.6 +/- 2.6 | 7.3 +/- 3.2    | 0.61 | 7.6 +/- 2.5  | 0.97 |
| <b>Donor status</b>                      |             |                |      |              |      |
| Alive                                    | 1           | 19             |      | 2            |      |
| Domino transplant                        | 0           | 1              | 0.46 | 0            | 0.43 |
| HBD                                      | 8           | 76             |      | 6            |      |
| NHBD                                     | 8           | 40             |      | 9            |      |

Table A3.1: Baseline characteristics of the RT cohort, NoRT<sub>all</sub> cohort, and NoRT<sub>matched</sub> cohort. Significant p-value (< 0.05) are in bold. BMI: Body mass index, DM: Diabetes mellitus, (N)HBD: (non-)heart-beating cadaveric donor, HBV: Hepatitis B virus, HCV: Hepatitis C virus, MELD: Model for end-stage liver disease, NET: Neuroendocrine tumour, RT: Radiotherapy, r-TAG: rabbit anti-lymphocytic serum. \* 3 missing data

In the RT cohort, 14 patients receive RT as a bridge therapy before liver transplantation for a localised hepatocarcinoma. For the three others, RT was part of the neoadjuvant treatment before transplant for an unresectable peri-hilar cholangiocarcinoma (Table A3.2).

| <b>Tumour and treatment characteristics</b>    | <b>RT cohort (n = 17)</b>       | <b>NoRT<sub>matched</sub> cohort (n = 17)</b> |
|------------------------------------------------|---------------------------------|-----------------------------------------------|
|                                                | <b>hepatocarcinoma (n = 14)</b> | <b>hepatocarcinoma (n = 16)</b>               |
| <b>T stage (TNM 8<sup>th</sup> edition)</b>    |                                 |                                               |
| T1a                                            | 5                               | 5                                             |
| T1b                                            | 2                               | 2                                             |
| T2                                             | 7                               | 9                                             |
| <b>BCLC stage</b>                              |                                 |                                               |
| 0                                              | 3                               | 2                                             |
| A                                              | 2                               | 5                                             |
| B                                              | 5                               | 1                                             |
| C                                              | 2                               | 7                                             |
| D                                              | 2                               | 1                                             |
| <b>Bridge therapy (before RT in RT cohort)</b> |                                 |                                               |
| NONE                                           | 4                               | 4                                             |
| Single TACE                                    | 2                               | 2                                             |
| Multiple TACE                                  | 5                               | 7                                             |
| Other modalities                               | 3                               | 3                                             |
| <b>RT scheme</b>                               |                                 |                                               |
| 6 x 6 Gy                                       | 1                               |                                               |
| 6 x 8 Gy                                       | 2                               |                                               |
| 5 x 8 Gy                                       | 4                               |                                               |
| 5 x 10 Gy                                      | 5                               |                                               |
| 3 x 15 Gy                                      | 1                               |                                               |
| 3 x 16 Gy                                      | 1                               |                                               |

|                                     | Cholangiocarcinoma<br>(n= 3) | Cholangiocarcinoma (n= 1) |
|-------------------------------------|------------------------------|---------------------------|
| <b>RT scheme</b>                    |                              |                           |
| 30 x 1,5 Gy (+<br>Boost of 15 Gy)   | 2                            |                           |
| 25 x 2 Gy                           | 1                            |                           |
| <b>Concomitant<br/>chemotherapy</b> |                              |                           |
| 5-Fluorouracile                     | 2                            |                           |
| Capecitabine                        | 1                            |                           |

*Table A3.2: Tumour and treatment characteristics of the matched cohorts. BCLC: Barcelona-Clinic Liver-Cancer, RT: Radiotherapy, TACE: Transarterial chemoembolisation.*

### 7.3.3.2. Acute cellular rejection

The early liver biopsy was missing for 3 patients in the RT cohort, 42 in the NoRT<sub>all</sub> cohort, and 4 patients in the NoRT<sub>matched</sub> cohort. Banff scores was significantly lower for the RT cohort (median: 2.5 [2 – 5.5]) compared to the NoRT<sub>all</sub> cohort (median: 5 [3.25 - 6],  $p = 0.043$ ). However, no significant difference was demonstrated after matching (median NoRT<sub>matched</sub> cohort: 4 [1 - 5],  $p = 0.62$ ). There were 3, 40 and 5 cases of moderate acute cellular rejection in the RT cohort, NoRT<sub>all</sub> cohort, and the NoRT<sub>matched</sub> cohort, respectively. The number of patients experienced severe acute cellular rejection were 1, 14, and 1 in the RT cohort, NoRT<sub>all</sub> cohort, and the NoRT<sub>matched</sub> cohort, respectively (Figure A3.1).

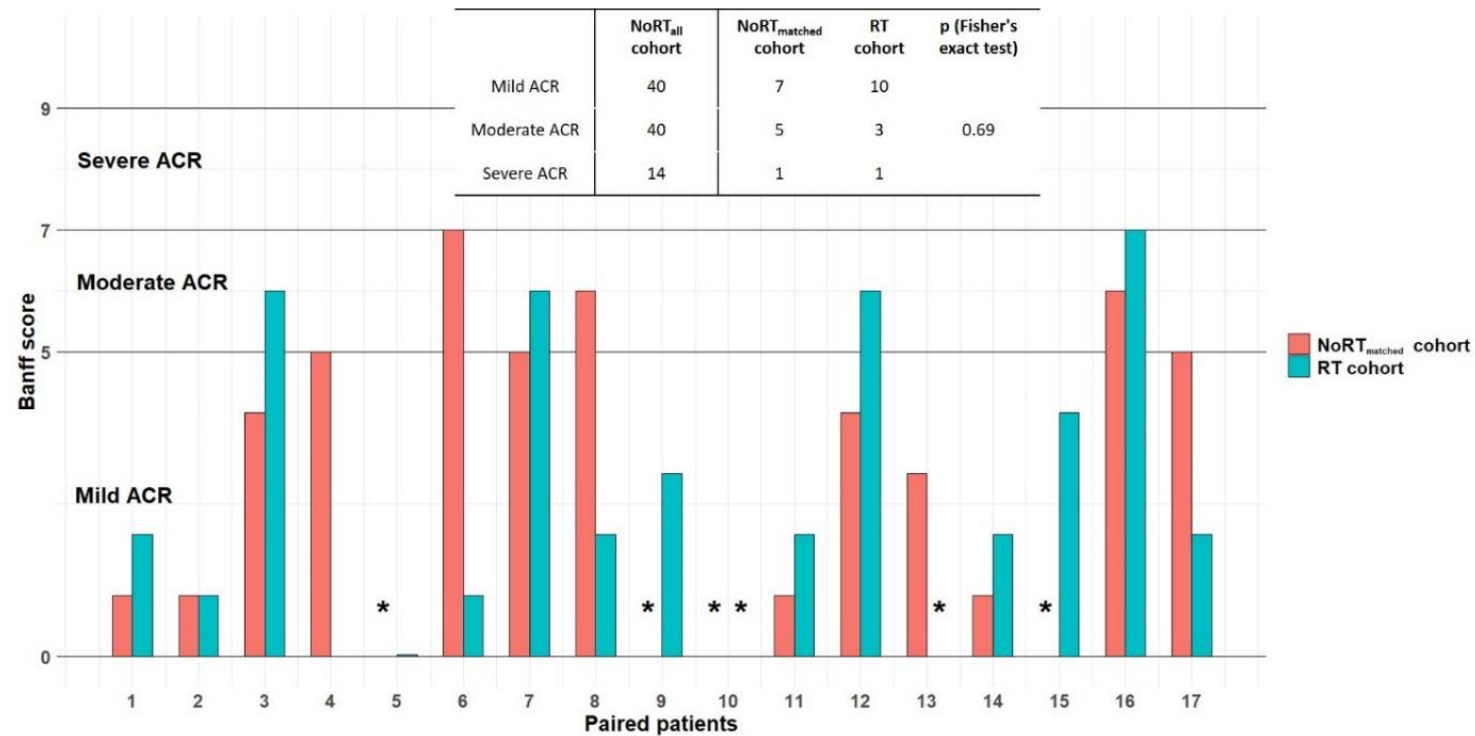


Figure A3.1: Acute cellular rejection for paired patients from the RT cohort and the NoRTmatched cohort. acute cellular rejection: acute cellular rejection, RT: Radiotherapy. \* Missing early liver biopsy.

Further immunosuppressive treatment was required in a total of 17 patients with moderate or severe acute cellular rejection. Among these 17 patients, two were in the RT cohort and two in the NoRT<sub>matched</sub> cohort. Total bilirubin levels were increased in eight out of ten patients with moderate or severe acute cellular rejection in the RT and NoRT<sub>matched</sub> cohorts (range 2.3 - 31.5 mg/dL). In most cases, total bilirubin normalised rapidly (7 - 189 days), except for one patient in the RT cohort who developed chronic graft rejection (> 6 months, Table A3.3).

| atient<br>identificatio<br>n number | Cohor<br>t          | Banff<br>score      | Rejection treatment | Maximum total bilirubin<br>level during acute<br>rejection (mg/dL) | Time from transplant<br>to bilirubin<br>normalisation (days) | Chronic<br>rejection<br>evolution | Follow-up<br>duration<br>(years) |
|-------------------------------------|---------------------|---------------------|---------------------|--------------------------------------------------------------------|--------------------------------------------------------------|-----------------------------------|----------------------------------|
| 1                                   | NoRT<br>matc<br>hed | 5 -<br>Mod<br>erate | None                | 5.5                                                                | 7                                                            | No                                | 5.1                              |
| 6                                   | NoRT<br>matc<br>hed | 7 -<br>Sever<br>e   | Tacrolimus increase | 4.9                                                                | 60                                                           | No                                | 3.5                              |
| 7                                   | NoRT<br>matc<br>hed | 5 -<br>Mod<br>erate | Tacrolimus increase | 8.8                                                                | 86                                                           | No                                | 2.9                              |
| 8                                   | NoRT<br>matc<br>hed | 6 -<br>Mod<br>erate | None                | 3.0                                                                | 8                                                            | No                                | 3.9                              |
| 16                                  | NoRT<br>matc<br>hed | 6 -<br>Mod<br>erate | None                | 2.6                                                                | 9                                                            | No                                | 4.1                              |
| 17                                  | NoRT<br>matc<br>hed | 5 -<br>Mod<br>erate | None                | 9.4                                                                | 169                                                          | No                                | 7.0                              |

|    |    |                     |                                                                                                  |                       |     |     |     |
|----|----|---------------------|--------------------------------------------------------------------------------------------------|-----------------------|-----|-----|-----|
| 25 | RT | 6 -<br>Mod<br>erate | Tacrolimus increased,<br>Addition of mycophenolate<br>mofetil,<br>methylprednisolone, and<br>ATG | 31.5                  | 265 | Yes | 4.0 |
| 27 | RT | 7 -<br>Sever<br>e   | None                                                                                             | No bilirubin increase |     | No  | 1.7 |
| 32 | RT | 6 -<br>Mod<br>erate | None                                                                                             | 2.3                   | 11  | No  | 1.9 |
| 33 | RT | 6 -<br>Mod<br>erate | Tacrolimus increase                                                                              | No bilirubin increase |     | No  | 2.3 |

*Table A3.3: Details of patients with moderate/severe acute rejection based on Banff score in the RT and the NoRTmatched cohorts. ATG: Antithymocyte globulin, RT: Radiotherapy.*



### 7.3.3.3. *Oncological outcomes*

Fourteen patients from the RT cohort, 110 from the NoRT<sub>all</sub> and 16 from the NoRT<sub>matched</sub> cohort were included in the survival analysis. The median follow-up was 4.4 years [2.2 – 6.3]. The 5-year overall survival was 62.1% in the RT cohort and was not significantly different compared to both the NoRT<sub>all</sub> cohort (76.0%,  $p = 0.52$ ) and the NoRT<sub>matched</sub> cohort (82.5%,  $p = 0.15$ ). The median overall survival was not reached for all cohorts at the time of the last follow-up (Figure A3.2A). Two patients in the RT cohort died prematurely following the liver transplantation (one due to haemorrhagic shock following hepatic artery dissection, one due to humoral graft rejection). In the NoRT<sub>all</sub> cohort, 13 patients died in the immediate aftermath of the graft (three haemorrhagic shock, two humoral graft rejection, two septic shock, two hepatic failure following hepatic artery thrombosis, one vasoplegic shock, one respiratory failure, and two undetermined causes) in which one death due to hepatic failure following hepatic artery thrombosis occurred in the NoRT<sub>matched</sub> cohort. The oncological progression caused two and eleven deaths in the RT cohort and the NoRT<sub>all</sub> cohorts, respectively (none in the NoRT<sub>matched</sub> cohort). The 5-year disease-free survival was 69.2% in the RT cohort and was significantly lower than the 5-year disease-free survival of 89.6% of the NoRT<sub>all</sub> cohort ( $p = 0.002$ ). However, after comparison with the NoRT<sub>matched</sub> cohort (5-year disease-free survival of 90.0%), this statistical difference with the RT cohort no longer existed ( $p = 0.05$ ). The median disease-free survival was not reached for all cohorts (Figure A3.2B). In the RT cohort, all oncological recurrences occurred within the first two years

following RT, while no recurrence occurred during this time interval in the NoRT<sub>matched</sub> cohort.

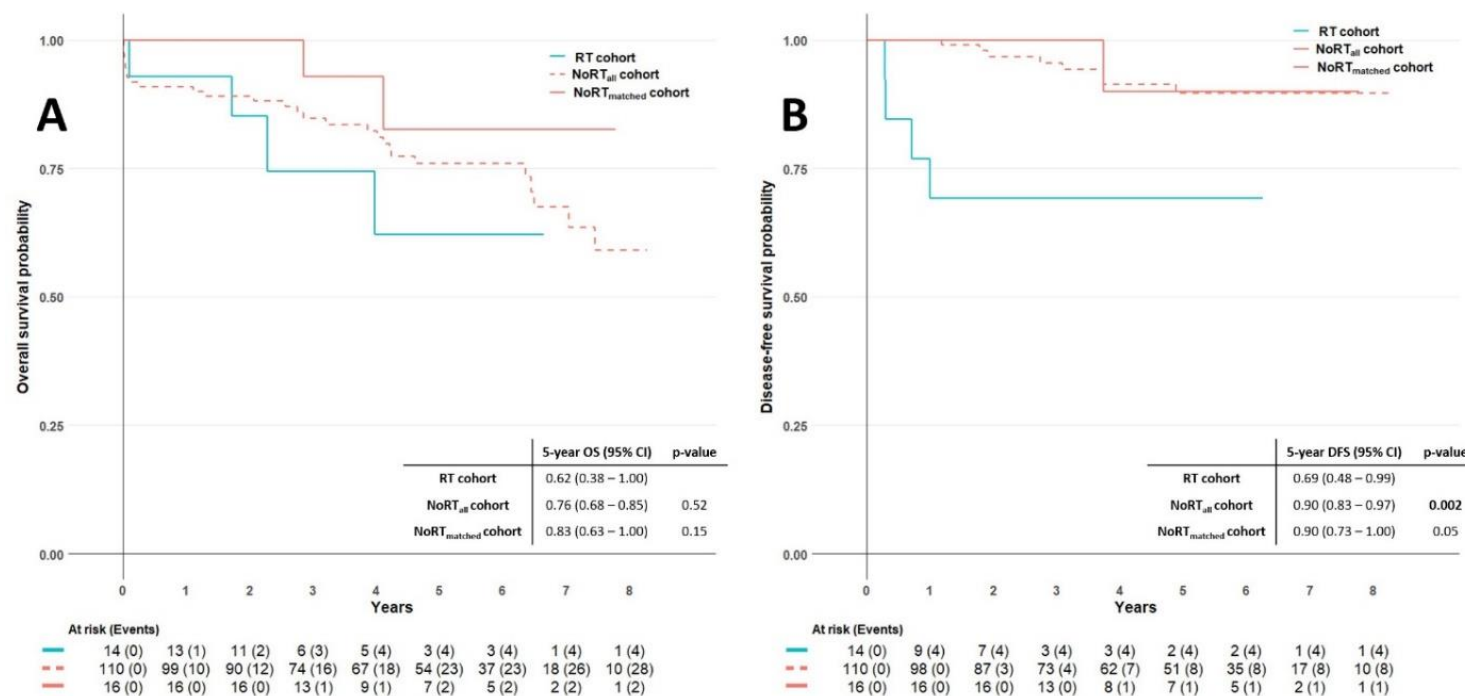


Figure A3.2: Overall survival (A) and disease-free survival (B) comparison between RT cohort, NoRTall cohort, and NoRTmatched cohort. CI: Confidence interval, DFS: Disease-free survival, OS: Overall survival, RT: Radiotherapy.

#### **7.3.4. Discussion**

In this study, a cohort of 17 patients who underwent RT before liver transplantation was compared with a paired cohort of patients who did not have RT before liver transplantation, obtained after propensity score-matching analysis. To our knowledge, this is the first study evaluating the impact of liver RT on acute cellular rejection. We showed that RT given before liver transplantation does not impact the occurrence nor the severity of acute cellular rejection, based on the Banff score on the early liver biopsy. None of the included patients required emergency liver transplantation or died because of acute cellular rejection.

RT can induce a local and systemic immune response that mainly involves T cells. Its effect on T cells is complex and depends on multiple factors, including the site and the dose of RT [758]. RT can activate T cells by releasing neoantigens from dying tumoral cells that will be processed by dendritic cells in the tumour-draining lymph nodes. T cells are then activated by these dendritic cells, migrate to distant tumoral sites, and can induce immunogenic cell death [204]. Additionally, following irradiation, tumoral cells generate damage-associated molecular patterns [759]. These damage-associated molecular patterns are similarly released in response to ischemia-reperfusion injury or infections that are associated with liver transplantation [751]. In liver transplantation, damage-associated molecular patterns can produce an adaptive immune reaction through activation of alloreactive T cells, resulting in a proinflammatory environment that favours transplant rejection [751]. RT can also induce systemic lymphopenia and, therefore, an

increase in the neutrophils-to-lymphocytes ratio (NLR), which can be long lasting and associated with poor oncological outcomes [760]. While preoperative lymphocyte count seems not to be correlated with acute cellular rejection in liver transplantation patients [761, 762], an increased preoperative NLR is associated with a higher rate of graft loss and poorer oncological outcomes in hepatocarcinoma patients [763, 764]. However, focusing only on T cells is probably inappropriate to establish the real impact of RT on acute cellular rejection in liver transplantation patients because both phenomena involve many other immune cells [25, 751].

Liver SABR is an alternative to other local bridge therapies before liver transplantation. It can be achieved with a low rate of toxicity, including mainly abdominal discomfort, nausea/vomiting and liver function worsening. Reported SABR-induced grade  $\geq 3$  adverse events are rare in this indication [513, 514, 765]. In our RT cohort, 5-year overall survival in hepatocarcinoma patients reached 62.1%, which is inferior compared to other series, in which 5-year overall survival ranged from 65% to 100% [513, 514, 765]. It is probably due to a selection bias related to our institutional policy reserving liver SABR as bridge therapy mainly when the patient has comorbidities that contraindicate other local bridge therapy, or when the tumour is not controlled after transarterial chemoembolisation or radiofrequency ablation. We report a 5-year disease-free survival of 69.2% in our RT cohort, which is also inferior compared to other published reports (74% – 100%) [513, 514, 765]. However, all of the four oncological recurrences in our series occurred during the first year following liver transplantation. It suggests a more advanced oncological disease in these

patients, who should not have been considered eligible for liver transplantation.

Our results have several limitations due to the retrospective design of this study and the small number of patients included in our RT cohort leading to a potential lack of power to highlight a differential effect between irradiated and non-irradiated patients. PSM analysis allows us to select patients in the NoRT<sub>all</sub> cohort that have similar baseline characteristics compared to the RT cohort for a more appropriate comparison between groups. With the rising role of RT as a bridge therapy before liver transplantation for hepatocarcinoma patients, further studies on larger populations will be required to confirm our results. Additionally, the heterogeneity in the RT indication and scheme (biological equivalent dose ranging from 57,6 Gy to 124,8 Gy in the RT cohort) prevents drawing definitive conclusions about the real impact of RT on acute cellular rejection after liver transplantation. Particularly, different RT fraction doses can have opposite effects on immune effectors [25, 766]. The systematic use of tacrolimus as immunosuppressive therapy and the systematic liver biopsy at around 7 days post-liver transplantation allows for reducing other bias linked to the surgery and immunosuppressive procedure.

### **7.3.5. Conclusion**

Liver RT can be given as a bridge therapy in hepatocarcinoma or as part of the neoadjuvant chemoradiotherapy for cholangiocarcinoma before liver transplantation. Despite its potential effect on the local and systemic

immune systems, liver RT has no impact on acute cellular rejection based on the Banff score reported in the early liver biopsy. Additionally, liver transplantation patients with liver RT before transplantation present similar overall survival and disease-free survival compared to a matched cohort of non-irradiated liver transplantation patients. Based on these results, and subject to analysis of larger cohorts, liver RT could be performed safely in patients requiring liver transplantation.

## 7.4. Appendix 4: Dealing with rectum motion during radiotherapy: how can we anticipate it?

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### Highlights

- Rectum motion is crucial to consider for pelvic radiotherapy
- Interfraction rectum motion is higher than intrafraction motion
- Rectum motion increases with radiotherapy session duration
- Rectum motion is higher when tumour-free and lower in the tumour-invaded part
- Rectum motion increase with the distance from the anorectal junction



## **Abstract**

### *Introduction*

Intra- and inter-fraction rectum motion is important for pelvic radiotherapy (RT). This study assesses how RT session duration, the presence or the absence of an intra-rectal tumour, and the distance from the Child-Pugh score impact rectal motion.

### *Materials and methods*

Analyses used cone-beam computed tomographies (CBCTs) from RT patients treated for rectal and prostate cancer. Three structures were evaluated: (1) the entire rectum in patients without a rectal tumour (Rectum<sub>Prostate</sub>); (2) the non-invaded portion (Rectum<sub>Rectum</sub>) and (3) the tumour-invaded portion (Rectum<sub>Tumour</sub>) in rectal cancer patients. Intrafraction motion was assessed using the Hausdorff distance 95% and the Mean distance-to-agreement between structures delineated on the first CBCT and the 2 subsequent CBCTs within a same RT session. Interfraction motion was quantified by comparing structures delineated on the planning-CT and the first CBCT of each session. Linear mixed model evaluated rectum motion in relation to time, tumour presence, and Child-Pugh score, respectively.

### *Results*

We included 10 patients with and 10 without rectal cancer, collecting 385 CBCTs. A significant correlation ( $p < 0.05$ ) between rectum motion and RT session duration was found. Intrafraction motion was significantly higher in prostate cancer patients (Rectum<sub>Prostate</sub> motion > Rectum<sub>Rectum</sub> and Rectum<sub>Tumour</sub>,  $p < 0.01$ ). For interfraction motion, only the mean distance to

agreement was significantly higher for Rectum<sub>Prostate</sub> ( $p < 0.05$ ). Motion increased significantly with Child-Pugh score for all three structures ( $p < 0.001$ ).

### *Conclusions*

Session duration, absence of a tumour, and Child-Pugh score are associated with larger intra- and interfraction rectal motion. This highlights the need for tailored RT treatment, including online-adaptive RT, to manage intra- and interfraction variations. Rectal motion should be handled differently for patients with prostate cancer and those with rectal cancer.

#### **7.4.1. Introduction**

Managing rectum motion is crucial for pelvic radiotherapy (RT) planning, as the rectum could be either part of the target volume in locally advanced rectal cancer (LARC) or an organ-at-risk, such as in prostate or cervix cancer [102, 115, 285, 767-769].

For LARC, a neoadjuvant radiotherapy (RT) and chemotherapy combination is increasingly recommended before surgery [102, 115, 285]. Recent trials with total neoadjuvant strategies have improved the progression-free survival in first line and, also, increased complete pathological response rates, questioning the need for surgery [103, 121, 287]. Interest in conservative treatments, such as “Watch and wait” approach has certainly risen since then. Given the demonstrated relationship between the RT dose to the primary tumour and the complete response rate, RT dose escalation is increasingly investigated for organ preservation [162, 770, 771]. However, delivering an external beam RT boost can be limited by the unavoidable dose to organs-at-risk such as the bladder and the small bowel [164]. Moreover, multiple uncertainties restrain this RT dose escalation, including the rectum tumour motion.

On the other hand, in pelvic RT for other malignancies, the rectum is an important organ-at-risk that must be preserved to avoid toxicities that can negatively impact the quality of life of the patient [767-769]. In these patients, personalised RT techniques that help to manage rectal motion, such as ART, offer an opportunity to reduce rectal toxicity [772, 773].

A comprehensive determination of the rectum motion would facilitate personalised external beam RT boost or rectum-sparing treatments, including online-adaptive RT (oART). It could allow the computation of an accurate planning target volume (PTV) margin to be added around the boost volume or a rectum planning organ-at-risk volume (PRV) for other pelvic RT location. oART can be performed with magnetic resonance imaging (MRI)-guided RT or cone-beam computed tomography (CBCT)-guided oART [194, 500, 774-776]. By using daily planning and online tracking of the tumour, MRI-guided RT has the potential to prevent geographic misses and excessive dose to organs-at-risk due to both intra- and inter-fraction tumour and rectum motion [774, 775]. CBCT-guided oART cope with the inter-fraction variations, but does not allow real-time tracking and, thus, cannot compensate for intra-fraction motion, which should be included in the PTV/PRV computation. However, these two technologies require substantial material resources and a dedicated team, making them less available. Therefore, integrating inter-fraction motion in PTV/PRV margin computation is essential for personalised RT when oART is unavailable.

Understanding the rectal motion is essential to provide tailored RT treatments. In this study, we analysed three factors that potentially impact the rectum motion on CBCTs performed during RT. First, we wondered if the rectum motion varied along a whole RT session. Second, we evaluated the impact of the presence of a tumour on the intra-fraction and inter-fraction rectum motion. Finally, we evaluated whether the motion of a segment of the rectum was greater according to its distance from the anorectal junction.

## **7.4.2. Methods**

### ***7.4.2.1. Patient selection***

Patients treated with RT for rectal cancer were prospectively included from January to July 2023. Children, patients with a previous pelvic RT, and patients with peritoneal carcinomatosis were excluded. A second cohort of patients without rectal cancer was created by retrospectively including patients treated for a prostate adenocarcinoma by stereotactic ablative RT (SABR) from December 2022 to September 2023. This monocentric study, conducted in accordance with the Helsinki declarations, was approved by the ethics committee of Cliniques Universitaires Saint-Luc (reference number: B4032022000061). Informed consent was provided by rectum cancer patients.

### ***7.4.2.2. RT procedure and CBCT acquisition***

In rectum cancer patients, a 3-mm slice thickness planning-CT (Aquilion LB, Canon medical systems corporation, Japan) was acquired in a supine position with a comfortably full bladder (300 ml of water 60 minutes before acquisition) and no rectum preparation. For prostate cancer patients, the planning-CT was also acquired in the supine position with the same bladder filling protocol. These patients had 2-mm slices acquired with additional immobilisation by a vacuum bag (Orfit, Belgium) and a rectum voidance protocol (rectal enema about an hour before CT acquisition if required).

For rectal RT, 25 Gy in 5 daily fractions was prescribed for short-course RT, and 45/54 Gy in 25 daily fractions for long-course RT to the pelvic lymph nodes according to the recommendations of Valentini et al. 2016 [161]. A simultaneous integrated boost was delivered to the primary tumour, extending to the entire adjacent rectal wall. A 7-mm and 10-mm isotropic PTV margin were used for the pelvic and boost volumes, respectively. For prostate stereotactic ablative RT, most patients received 29 Gy in 5 fractions every other day prescribed to the seminal vesicles with a 7-mm isotropic PTV margin. A simultaneous integrated boost of 35 Gy was delivered to the whole prostate with a 5-mm PTV margin. One patient received 36 Gy in 6 weekly fractions. Planning was performed on the Raystation planning system (clinical versions 9B and 12A, RaySearch Laboratories, Stockholm, Sweden). All patients were treated in one institution on two linear accelerators (Halcyon® and Ethos®, Varian a Siemens Healthineers Company, Palo Alto, Calif., USA) using equidistant field intensity modulated RT or volumetric modulated arc therapy.

Three CBCTs per session were acquired for image-guided RT. For SABR prostate patients, the acquisition of these three CBCTs was part of our institutional standard-of-care due to hypofractionated high-dose delivery. For rectum patients, the second and third CBCTs were obtained solely for the purpose of this analysis. For both groups of patients, the first CBCT (CBCT1) is acquired at the beginning of the session, allowing an initial co-registration with the planning-CT. This co-registration is based on the bony pelvis for rectum patients and radiopaque gold markers for prostate patients. Based on this co-registration, translational table displacements in

the three axes can be done before the acquisition of a second CBCT (CBCT2), which was acquired just before RT delivery. A second rigid co-registration with the planning-CT is then performed, to ensure that there is no major intra-fraction variation, and supplementary table displacements are allowed. After the RT treatment, a third CBCT (CBCT3) is finally acquired and co-registered with the second CBCT, in order to evaluate the intrafraction anatomical variations. In this trial, for short-course rectal RT and prostate cancer patients, these three CBCTs were acquired at each session (totalling 15 CBCTs per patient), though there were some missing images in the retrospective prostate cancer group. For long-course rectum RT patients, these three CBCTs were acquired during the five first sessions and then during the first RT session of each following week, totalling 27 CBCTs per patient.

#### *7.4.2.3. Motion evaluation*

All our analyses were conducted offline in a test version of the Raystation planning system dedicated to research, in which all the CBCTs were exported, cleaned of any co-registration information. For the evaluation of the interfraction motion, the CBCT1 was co-registered to the planning-CT, with a bone-based rigid co-registration. For the intrafraction motion evaluation, we compared the CBCT2 & CBCT3 to the CBCT1. The CBCT2 displacements to align with the planning-CT and the CBCT3 displacements to align with CBCT2, which were made during the clinical RT session, were ignored for these analyses. In other terms, CBCT1, CBCT2, and CBCT3 were

acquired in the same frame of reference, and no additional displacements or co-registrations were performed between them.

The rectum was manually delineated on each (CB)CT by one operator for all patients according to the guidelines of Mir et al., 2020 [777]. For rectal RT patients, a separate volume encompassing the circumferential rectal wall was delineated at each CBCT slice where the tumour was visible, using a co-registered planning-MRI. The anal canal wall was also included if the tumour extended inside. Therefore, we compared three groups of rectal structures: (1) the whole rectum of prostate cancer patients in which no tumour was present ( $\text{Rectum}_{\text{Prostate}}$ ), (2) the whole rectum of rectum cancer patients in which a tumour was present (including the tumoral part,  $\text{Rectum}_{\text{Rectum}}$ ), and (3) the tumoral rectal wall in rectum cancer patients ( $\text{Rectum}_{\text{Tumour}}$ ).

The first analysis evaluated rectal motion as a function of time on a single treatment session timescale. For that purpose, we compared the motion of the rectum between CBCT1 and CBCT2 of the day to the motion between CBCT1 and CBCT3. No initial registration was made between CBCTs of the same session. We used the Hausdorff distance 95% (HD95) and the mean distance-to-agreement to quantify this motion. The HD95 is a surrogate of the maximal distance between overlapping structures, and the mean distance-to-agreement quantifies the average distance between two structures. These values were reported for the three groups of volumes ( $\text{Rectum}_{\text{Prostate}}$ ,  $\text{Rectum}_{\text{Rectum}}$ , and  $\text{Rectum}_{\text{Tumour}}$ ).

The second analysis compared rectal motion based on the presence or absence of a tumour. Intra- and interfraction HD95 values of  $\text{Rectum}_{\text{Prostate}}$ ,



Rectum<sub>Rectum</sub>, and Rectum<sub>Tumour</sub> were compared. For interfraction motion evaluation, volumes delineated on CBCT1 were compared with those of the planning-CT after bone-based rigid co-registration. To assess intrafraction motion, volumes delineated on CBCT3 (or, if unavailable, CBCT2) were compared with those of the CBCT1 from the same day without co-registration between CBCTs (Figure A4.1).

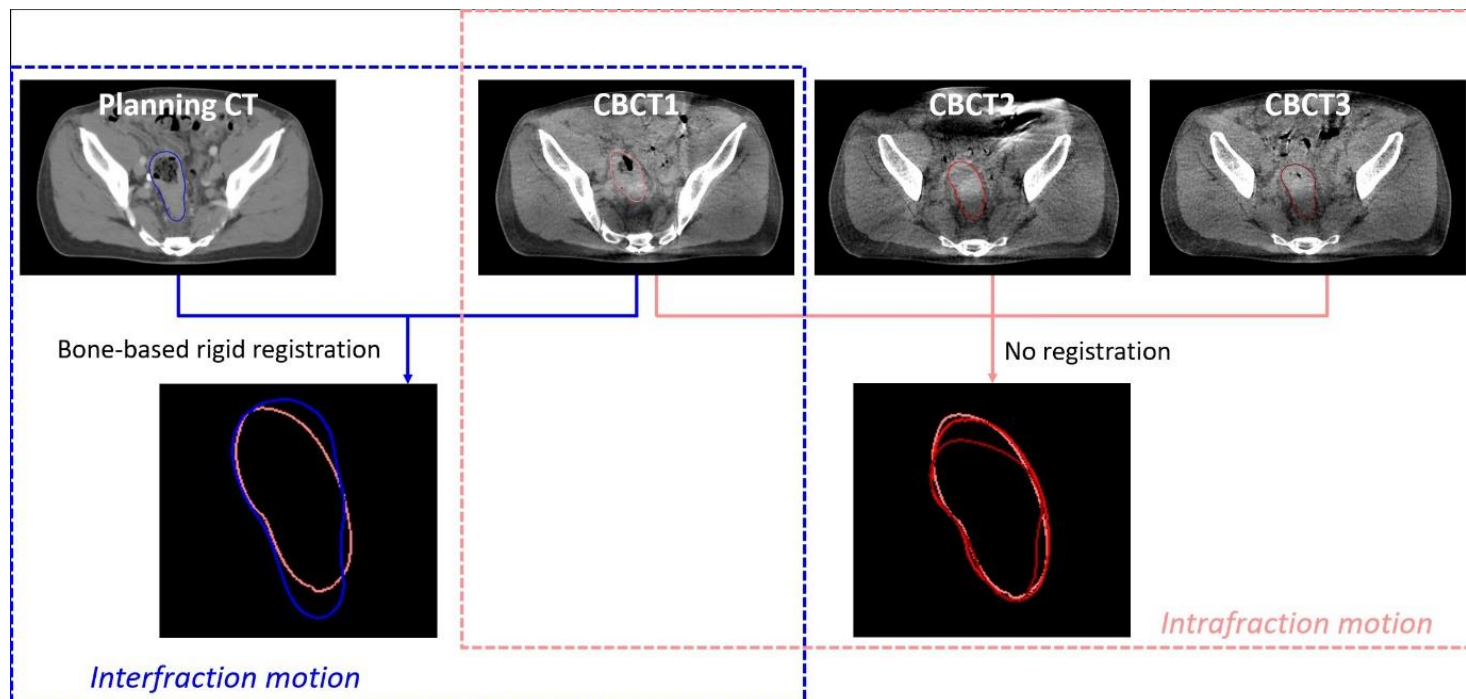


Figure A4.1: Intrafraction and interfraction rectum motion evaluation. For intrafraction motion evaluation, rectum volumes on the three CBCTs of the session have been compared without any co-registration. For interfraction motion evaluation, rectum volumes on planning-CT and the first CBCT of the session have been compared after a bone-based co-registration between both images. (CB)CT: (Cone-beam) computed tomography.

The third analysis evaluated whether rectum motion amplitude varied with distance from the anorectal junction. Intra- and interfraction motion were quantified for Rectum<sub>Prostate</sub>, Rectum<sub>Rectum</sub>, and Rectum<sub>Tumour</sub> at each CBCT slice level using the HD95. Unlike the first analyses comparing three-dimensional volumes, this comparison was made between sequential two-dimensional structures along the entire rectum. As opposed to the first analyses, here, Rectum<sub>Tumour</sub> slices were excluded from the Rectum<sub>Rectum</sub>. For each of the Rectum<sub>Prostate</sub>, Rectum<sub>Rectum</sub>, or Rectum<sub>Tumour</sub> slices, the distance from the anorectal junction was calculated by multiplying the CBCT slice thickness by the number of slices separating it from the first section of the rectum.

#### *7.4.2.4. Statistical analysis*

For each analysis (except the first one, aiming to evaluate the existence of a correlation between time and rectum motion), we reported the mean value of HD95 and mean distance-to-agreement over all sessions per patient. The distribution of HD95 and mean distance-to-agreement values was reported as the median [interquartile range] since they had a non-Gaussian distribution. The correlation between time and motion was performed using a linear mixed effect model where time was a fixed effect. Since multiple observations per patient were obtained, a linear mixed model was used with patients as the random factor when analysing the comparison between Rectum<sub>Prostate</sub>, Rectum<sub>Rectum</sub>, and Rectum<sub>Tumour</sub>, the comparison between intra- and interfraction, and the correlation between distance from the

anorectal junction and motion. In order to investigate the difference between categorical variables, post-hoc pairwise comparisons were performed with p-value adjustments for multiple comparison using the Benjamini-Hochberg method. A p-value < 0.05 was considered significant. HD95 and mean distance-to-agreement values were obtained using a homemade Python (version 3.10) script on Visual Studio Code (version 1.18.1). All data and statistical analyses were performed on RStudio (R version 4.2.1) using the “tidyverse”, “lme4”, and “ggpubr” packages.

### **7.4.3. Results**

#### **7.4.3.1. Patients**

We included 10 rectum cancer and 10 prostate cancer patients (Table A4.1). In the rectum cancer group, one patient underwent a short-course RT, and nine patients were treated with long-course rectal RT combined with chemotherapy for locally advanced or metastatic rectal adenocarcinoma. Eighty-six sessions with three CBCTs each were performed in the rectum cancer group. In the prostate cancer group, there were 35 sessions with three CBCTs and 11 with two CBCTs each. In the rectum cancer group, liver, lung, and lymph node metastases were found in 2, 1, and 2 patients. One prostate cancer patient had lymph node metastases. All metastatic patients had systemic disease control during RT of the primary tumour. No patient presented symptoms from their metastatic disease nor liver dysfunction.

|                                               | Prostate cancer patients | Rectum cancer patients |
|-----------------------------------------------|--------------------------|------------------------|
| <b>N</b>                                      | 10                       | 10                     |
| <b>Gender</b>                                 |                          |                        |
| Male                                          | 10                       | 9                      |
| Female                                        | 0                        | 1                      |
| <b>Age (mean +/- SD)</b>                      | 69.4 +/- 8.2             | 59.5 +/- 14.4          |
| <b>Pelvic surgery history</b>                 | 2                        | 1                      |
| <b>Inflammatory bowel disease history</b>     | 0                        | 0                      |
| <b>T stage</b>                                |                          |                        |
| Tx                                            | 1                        | 0                      |
| T1                                            | 3                        | 0                      |
| T2                                            | 5                        | 0                      |
| T3                                            | 1                        | 7                      |
| T4                                            | 0                        | 3                      |
| <b>N stage</b>                                |                          |                        |
| N0                                            | 9                        | 1                      |
| N1                                            | 1                        | 2                      |
| N2                                            | 0                        | 7                      |
| <b>M stage</b>                                |                          |                        |
| M0                                            | 9                        | 7                      |
| M1                                            | 1                        | 3                      |
| <b>Medication during RT</b>                   |                          |                        |
| Antispasmodic                                 | 0                        | 1                      |
| Opioid                                        | 0                        | 2                      |
| <b>Number of sessions with multiple CBCTs</b> | 46                       | 86                     |
| <b>Number of CBCTs</b>                        | 127                      | 258                    |

Table A4.1: Patient characteristics. CBCT: Cone-beam computed tomography, SD: Standard deviation

#### 7.4.3.2. *The impact of time*

In rectum RT patients, the median time elapsed from CBCT1 to CBCT2 and CBCT3 was 2.6 minutes [range: 2.2 – 3.3] and 6.9 minutes [6.0 – 7.9], respectively. For prostate cancer patients, this median time was 3.9 minutes [2.9 – 5.1] and 10.7 minutes [10.0 – 12.1], respectively. A significant positive correlation was found between time and increased motion in all groups (Rectum<sub>Prostate</sub>, Rectum<sub>Rectum</sub>, and Rectum<sub>Tumour</sub>;  $p < 0.05$ ). This correlation was the most pronounced for HD95 of Rectum<sub>Prostate</sub> ( $\beta = 0.61$ , Figure A4.2).

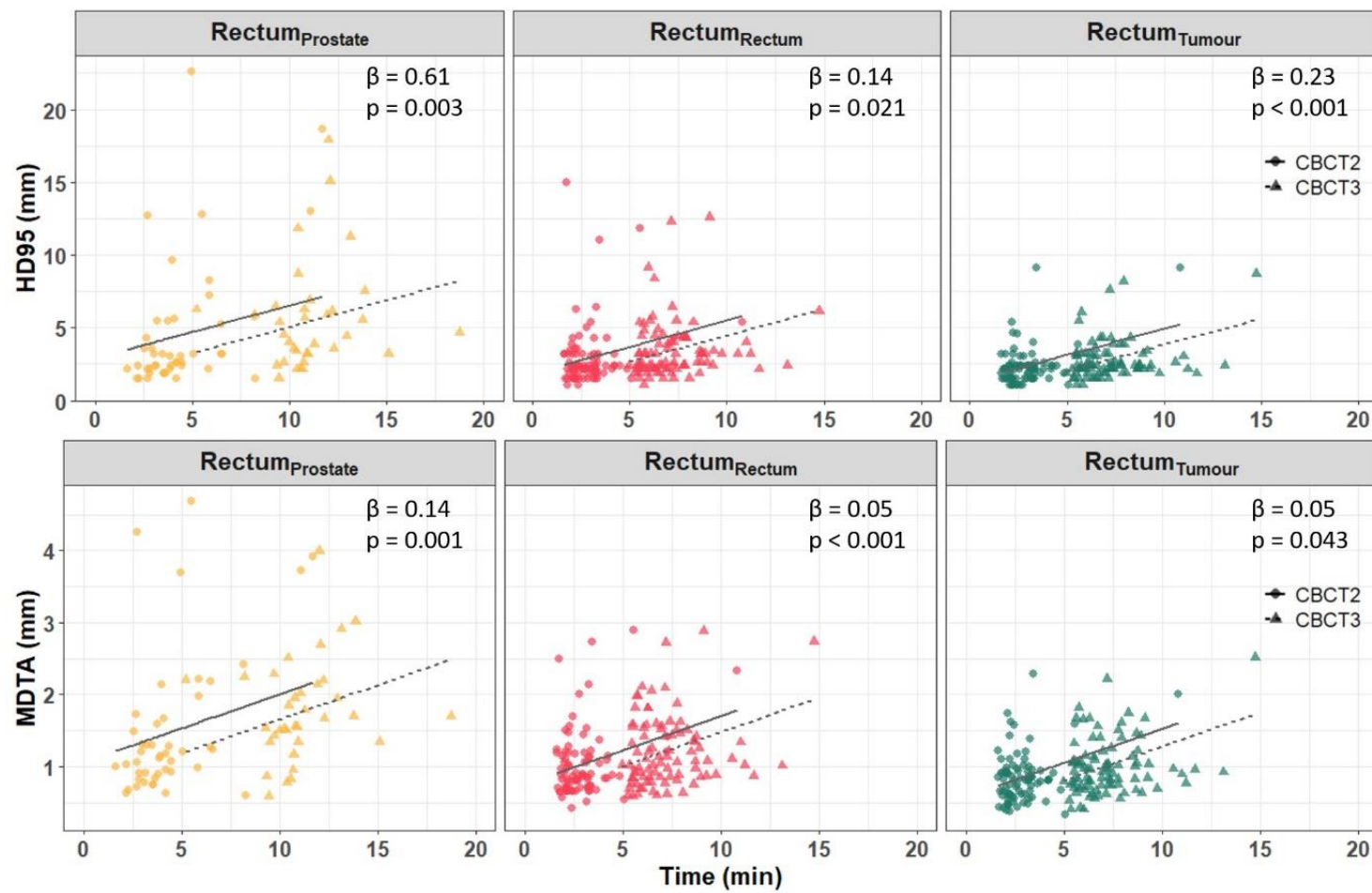


*Figure A4.2: Rectum motion increased with time. All three groups of structures (RectumProstate, RectumRectum, and RectumTumour) demonstrated a significantly motion increase during the duration of a radiotherapy session. This was assessed by the HD95 and mean distance-to-agreement between the same structures delineated on each cone-beam computed tomography of a session. Grey line: linear regression line. Linear mixed-effect model, p-value < 0.05 is significant. HD95: Hausdorff distance 95%, MDTA: Mean distance-to-agreement.*



At the time of the CBCT2, rectal motion was significantly smaller than at CBCT3 for all Rectum<sub>Rectum</sub> and Rectum<sub>Tumour</sub>, demonstrating a progressive increase of the motion over time (Table A4.2).

| Volumes                    | Metric    | CBCT2           | CBCT3           | p-value |
|----------------------------|-----------|-----------------|-----------------|---------|
| Rectum <sub>Prostate</sub> | HD95 (mm) | 3.7 [2.4 – 6.5] | 5.1 [4.4 – 7.3] | 0.32    |
|                            | MDTA (mm) | 1.4 [1.0 – 1.9] | 1.8 [1.6 – 2.0] | 0.13    |
| Rectum <sub>Rectum</sub>   | HD95 (mm) | 2.7 [2.5 – 3.3] | 3.3 [2.9 – 4.0] | 0.027   |
|                            | MDTA (mm) | 1.0 [1.0 – 1.1] | 1.2 [1.1 – 1.3] | 0.006   |
| Rectum <sub>Tumour</sub>   | HD95 (mm) | 2.3 [2.1 – 2.5] | 2.6 [2.3 – 3.2] | 0.037   |
|                            | MDTA (mm) | 0.9 [0.8 – 0.9] | 1.0 [0.9 – 1.1] | 0.010   |

*Table A4.2: Evolution of intrafraction rectal wall motion from the CBCT1 to the CBCT2 and to the CBCT3. For Rectum<sub>Rectum</sub> and Rectum<sub>Tumour</sub>, the motion of the rectum was significantly higher at the time of the CBCT3 than the CBCT2. The motion was quantified by reporting the HD95 and MDTA (median [interquartile range], in mm) between CBCT1 volumes and CBCT2/CBCT3 volumes. Wilcoxon paired test, p-value < 0.05 is significant. CBCT: Cone-beam computed tomography, HD95: Hausdorff distance 95%, MDTA: mean distance-to-agreement.*

#### 7.4.3.3. The impact of tumour presence

For intrafraction motion assessment, the median HD95 values were 5.9 mm [4.0 – 7.1], 3.7 mm [3.1 – 4.2], and 2.8 mm [2.5 – 3.3], and the median mean distance-to-agreement values were 1.9 mm [1.6 – 2.1], 1.3 mm [1.2 – 1.3], and 1.0 mm [0.9 – 1.2], for the Rectum<sub>Prostate</sub>, the Rectum<sub>Rectum</sub>, and the Rectum<sub>Tumour</sub>, respectively. Intrafraction motion was higher for Rectum<sub>Prostate</sub>, followed by the Rectum<sub>Rectum</sub>, with the Rectum<sub>Tumour</sub> showing the smallest variations (Figure A4.3A).

For interfraction motion, the median HD95 values were 15.7 mm [12.4 – 23.9], 11.3 mm [10.2 – 17.9], and 12.0 mm [9.0 – 13.3], and the median mean distance-to-agreement values were 4.6 mm [4.4 – 5.7], 3.8 mm [2.9 – 4.5], and 3.2 mm [2.5 – 4.0], for the Rectum<sub>Prostate</sub>, the Rectum<sub>Rectum</sub>, and the Rectum<sub>Tumour</sub>, respectively. Similarly, although less significantly than intrafraction motion, interfraction motion was greater for Rectum<sub>Prostate</sub> and smaller for Rectum<sub>Tumour</sub> (Figure A4.3B).

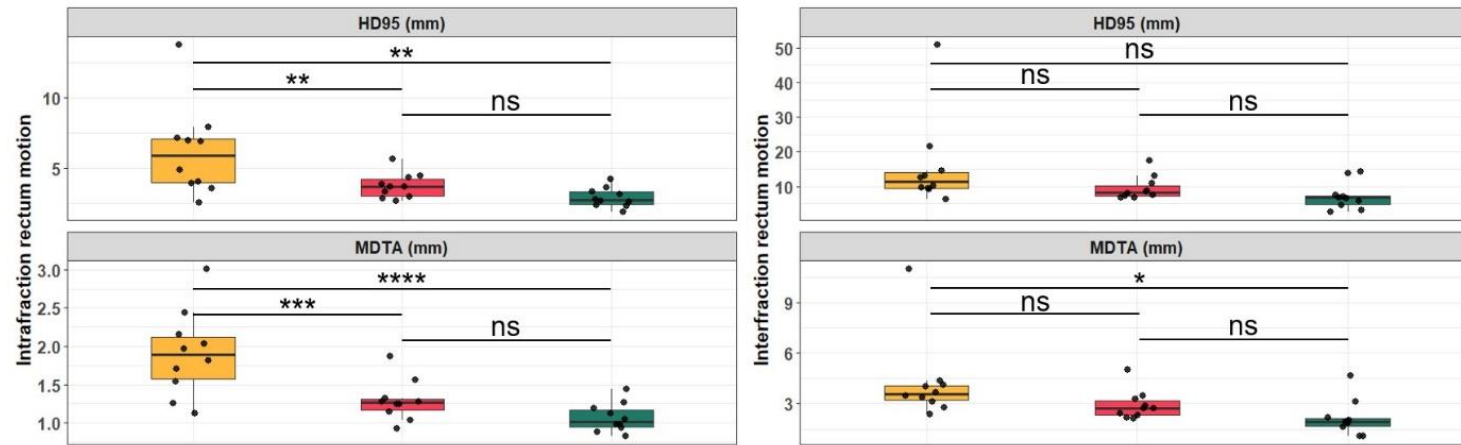


Figure A4.3: Intrafraction (A) and interfraction (B) motion of the rectal wall in prostate SABR patients, the rectum in rectum RT patients, and the tumoral rectal wall in rectum RT patients. Scales in the ordinate axes are different between intrafraction and interfraction motion. Linear mixed-effect model,  $p$ -value  $< 0.05$  is significant. ns: not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . HD95: Hausdorff distance 95%, MDTA: Mean distance-to-agreement, ns: Not significant, RT: Radiotherapy, SABR: Stereotactic ablative radiotherapy.

For all three groups of structures, interfraction motion was significantly higher than intrafraction motion (Figure A4.4).

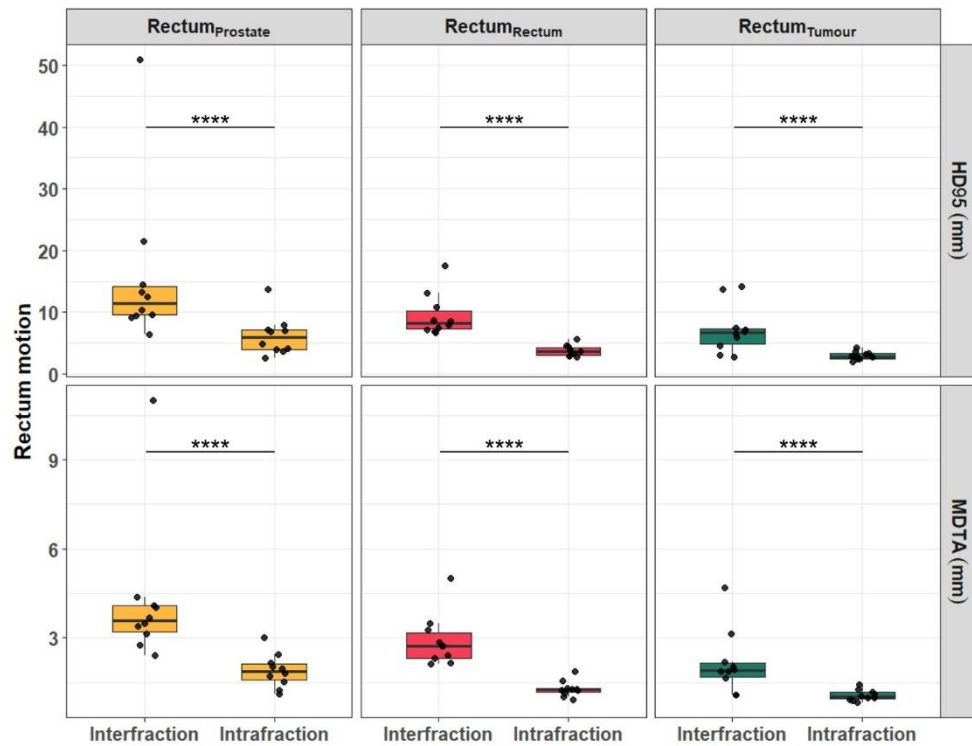


Figure A4.4: Comparison of the interfraction and intrafraction motion of the rectum for the three groups of structures. Linear mixed-effect model,  $p$ -value  $< 0.05$  is significant. \*\*\*\*  $p < 0.0001$ . HD95: Hausdorff distance 95%, MDTA: Mean distance-to-agreement.

#### *7.4.3.4. The impact of distance from the anorectal junction*

Linear mixed effect models demonstrated a significant correlation between an increased distance from the anorectal junction and an increase in intrafraction motion of the rectum ( $p < 0.001$ , Figure A4.5A). Similar results were demonstrated for the interfraction motion analysis ( $p < 0.001$ , Figure A4.5B). The fixed-effect regression coefficients between interfraction rectum motion and distance from the anorectal junction were the highest for Rectum<sub>Prostate</sub>, followed by Rectum<sub>Rectum</sub>, and, then Rectum<sub>Tumour</sub> (HD95 fixed-effect regression coefficient  $\beta = 1.81, 0.97$ , and  $0.77$ , and mean distance-to-agreement  $\beta = 0.85, 0.24$ , and  $0.19$ , respectively) so was the correlation with interfraction motion (HD95  $\beta = 0.55, 0.27$  and  $0.31$ , and mean distance-to-agreement  $\beta = 0.17, 0.06$  and  $0.08$ , respectively).

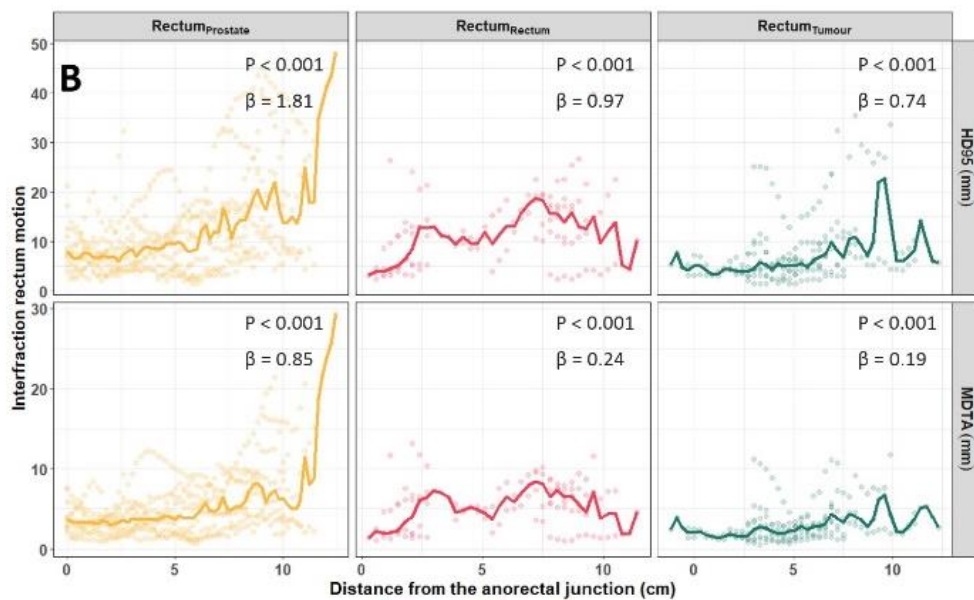
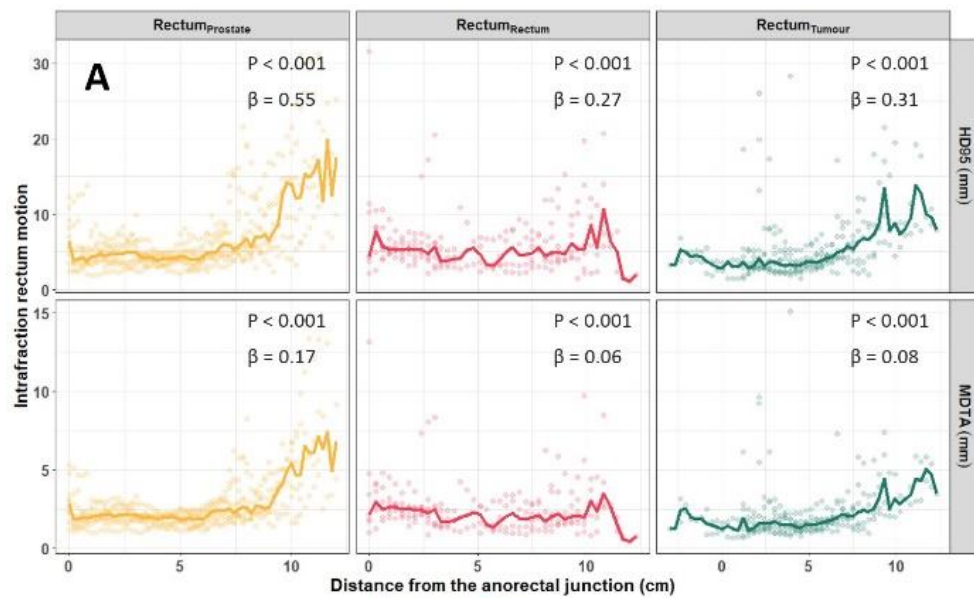


*Figure A4.5: Intrafraction (A) and interfraction (B) rectum motion increased with the distance from the anorectal junction for the RectumProstate, and RectumTumour. This was assessed by the HD95% and MDTA between the same structures delineated on CBCT. This correlation was higher for interfraction motion and for prostate cancer patients based on the fixed-effect regression coefficient  $\beta$ . Scales in the ordinate axes are different between intrafraction and interfraction motion. Coloured line: median value at each slice of the CBCTs, Grey line: linear regression line. Linear mixed-effect model,  $p$ -value  $< 0.05$  is significant. CBCT: Cone-beam computed tomography, HD95: Hausdorff distance 95%, MDTA: Mean distance-to-agreement. (Rectum image adapted from "Slagter, R. (n.d.). Drawing external and internal anal sphincter. AnatomyTOOL. <https://anatomytool.org/content/slagter-drawing-external-and-internal-anal-sphincter-no-labels>")*

#### **7.4.4. Discussion**

image-guided RT enhances the accuracy of RT delivery, potentially reducing toxicity and enabling dose escalation [191]. Daily CBCTs remain one of the most common image-guided RT strategies in pelvic cancer. However, comprehensive image-guided RT knowledge is necessary before implementing more complex techniques like oART. CBCT-guided or MRI-guided RT is promising but not yet widespread due to the need for substantial technical, financial, and human resources [187, 595]. In this study conducted on a total of 385 CBCTs, we analysed three factors impacting rectal motion during RT: session duration, tumour presence, and distance from the anorectal junction.

We found a positive correlation between rectum motion and RT session duration. Rectum intrafraction motion was significantly greater at CBCT3

than at CBCT2 in Rectum<sub>Rectum</sub> and Rectum<sub>Tumour</sub>. This was also observed in a cine-MRI study by Kleijnen et al., 2016, including 16 patients with a rectal tumour. They reported an average tumour motion within one minute of 2.3 mm (assessed by a metric similar to HD95). Most motion variation occurred between 1 and 18 minutes after reference image acquisition [166]. This is important for CBCT-guided oART, where average session durations (patient on couch) were 26 minutes for rectal and 17.5 minutes for prostate cancer RT [194, 778]. Reducing session time should be a key goal for pelvic oART to minimise rectum motion uncertainties and, therefore, PTV/PRV margins. Intrafraction motion was significantly lower than interfraction motion. This finding supports the use of oART that eliminates interfraction uncertainties and, thus, could reduce PTV/PRV margins compared to conventional RT. In the previously cited study of Kleijnen et al., 2016, a significant reduction of the PTV margin was achieved when only intrafraction tumour motion was considered, particularly with shorter session duration. This was no more applicable if the session duration was higher than 18 minutes [166]. PTV margins for interfraction motion compensation often exceed 1 cm but can be reduced to 5-8 mm using CBCT- or MRI-guided oART, eliminating interfraction motion[194, 292, 303, 428].

To our knowledge, this is the first study comparing rectum motion between patients with (Rectum<sub>Rectum</sub>, and Rectum<sub>Tumour</sub>) and without a rectal tumour (Rectum<sub>Prostate</sub>). Our results indicate that tumour presence decreases the rectum motion. Both intra- and interfraction rectum motions were higher for Rectum<sub>Prostate</sub> compared to Rectum<sub>Rectum</sub>. These results should lead radiation oncologists to adapt their strategies of rectum motion



management to the type of RT treatment, especially considering emerging RT treatments including prostate SABR and rectum dose escalation for organ preservation purpose [162, 770, 771, 779]. For example, margins can be used to limit rectal toxicity for prostate SABR patients (i.e. PRV) or to ensure correct target volume coverage in rectum patients (i.e. PTV). Our results suggest therefore that the uncertainties linked to rectal motion are lower for rectum cancer compared to prostate cancer patient and, thus, the rectum PTV used for rectum cancer patient should be smaller than the rectum PRV in prostate SABR patients.

The main limitations of our study are related to the heterogeneity between prostate cancer and rectum cancer patients. First, the preparation differed between the prostate SABR and the rectum RT groups. In the rectum RT group, no rectal preparation was recommended to avoid additional rectal irritation, while in the prostate SABR group, a strict rectum voidance protocol (bowel movement one hour before the RT session, using an enema if necessary) was required to minimise uncertainties linked to rectal volume variations during RT delivery. The impact of laxatives, enemas, and other preparation strategies (probiotics, adapted diet, or mechanical rectal evacuation) on interfraction motion remains debated, while for intrafraction motion, data are scarce [424, 425]. If there was an impact to consider, rectal emptying strategies would tend to reduce the variability of rectal volume and, consequently, its motion. In a study of Hoogeman et al., 2015, the rectum volume observed in the planning-CT was correlated with its interfraction variation, suggesting that voidance strategies could reduce interfraction motion [780]. Similarly, the use of enema during prostate RT

has the potential to limit prostate interfraction motion [781]. Additionally, in a different context, MRI studies have evaluated strategies to optimise image acquisition. Among these, enemas may improve or, at worst, have no impact on rectum motion artefacts [782, 783]. Extrapolating these data to our analysis's context, enemas and, more generally, rectal emptying protocols seem to have only a limited impact on rectal motion and, at worst, would tend to reduce rectal movement in our Rectum<sub>Prostate</sub> patients. Therefore, our conclusion about greater rectal motion in patients with prostate cancer compared to those with rectal cancer seems reinforced. Though, these results might be contra-intuitive for the prostate cohort, since rectal voidance protocols were built to reduce rectum, and thereby prostate motion during (stereotactic) radiotherapy. However, caution should be paid to the fact that this trial compared a rectal patient cohort to a prostate patient cohort. It did not focus on prostate cancer patients only and therefore did not compare cohorts of prostate SABR patients, and more specifically one with and one without voidance protocol. No conclusion can thus be made in that extend. Man should keep in mind that these results do not discriminated rectal voidance protocols recommended for prostate (stereotactic) radiotherapy. Second, CBCT2s were acquired later in prostate cancer patients compared to rectum cancer patients (median: 2.6 versus 3.9 minutes, respectively), as were CBCTs3 (median: 6.9 versus 10.7 minutes), potentially overestimating the rectum motion in prostate SABR patients. This was due to a longer image-guided RT time, requiring a validation of a radiation oncologists in prostate cancer patients, while, in rectum cancer patients, the whole treatment was performed by the radiation therapists.

Additionally, the dose per fraction and the delivered monitor units were higher in prostate cancer patients, leading to a higher RT session duration. We did not try to replicate the timing between CBCTs of prostate cancer patients in the prospective cohort of rectum cancer patients because of logistical implications (prolonged treatment slot, absence of patient benefit, and patient discomfort...) and to reproduce clinical conditions where multiple CBCTs are not acquired in rectum cancer patients. Third, three patients in the rectum RT group have taken opioids or antispasmodic medications during RT, which can potentially reduce gastrointestinal motion, while none of the prostate group patient were treated with those drugs. Constipation is a well-known adverse effect of opioid drugs and is related to prolonged colonic transit time, suggesting a reduction in rectal movement. However, the specific effect on the rectal segment remains debated [784-786]. Similarly, the effect of antispasmodic drugs on rectum motility seems to be limited [787]. Fourth, three and one patients had visceral metastases in the rectum and prostate cancer groups, respectively. None of them had peritoneal carcinomatosis or metastatic-induced liver failure, two conditions that can impact gastrointestinal peristalsis [788, 789]. However, whether other metastases can have a systemic impact on rectal motion is unknown; this deserves further investigation. Fifth, both the whole RT treatment duration and the volume of irradiated patients are smaller for prostate cancer patients compared to rectum cancer patients. Alexander et al., 2023 have demonstrated that the rectum volume variation and prostate intrafraction motion is similar in prostate cancer patient treated by either 5- or 20-fractions suggesting that the entire duration of the

RT treatment have no impact rectum motion [790]. In rectum cancer patients, the rectum volume is stable or tends to decrease along the RT course, but extrapolation of these results to inter/intrafraction rectal motion variation has not been demonstrated [296, 312]. Since bowel irradiation increases bowel mobility, reduces intestinal transit time, and is associated to diarrhoea, our results about a higher rectal motion in prostate cancer compared to rectal cancer patients seems to be reinforced [791-794]. To obtain definitive confirmation that rectum motion is reduced in rectal cancer patients compared to prostate cancer patients, further investigations that control these factors of heterogeneity are required.

Both intra- and interfraction motion of the Rectum<sub>Tumour</sub> increased with distance from the anorectal junction, consistent with Chong et al., 2011. They evaluated the interfraction motion of the rectum in 16 patients treated for a rectal adenocarcinoma based on an anatomical rectum division based in upper (0 - 5 cm from inferior border of L5), mid (5.1 – 10 cm), and lower rectum (> 10 cm). The upper rectum motion was significantly higher than that of the mid and the lower rectum. They recommended a PTV margin that accounts for setup and interfraction uncertainties, with to 1.76 cm in the anterior direction for the upper rectum [296]. Similar findings suggesting a higher interfraction motion of the upper rectum were demonstrated in other studies [294, 402, 795]. Our results found also an increased intrafraction motion with distance from the anorectal junction in the Rectum<sub>prostate</sub> group, but, surprisingly, not in the Rectum<sub>Rectum</sub> group. This was possibly due to our methodology that excluded the tumours from the whole rectum structure for the Rectum<sub>Rectum</sub> group.

This study has other limitations, such as the retrospective image collection in prostate SABR patients and the small number of patients. The high number of CBCTs included in this analysis could, however, ensure the robustness of the results for each patient. Also, the metrics used for motion quantification, HD95 and mean distance-to-agreement, are sensitive to the delineation variation of rectal structures. To reduce this impact, all volumes were delineated by one single operator. Additionally, we quantified only three factors that influence rectal motion, which should be integrated in the PTV/PRV margin or the image-guided RT workflows to guide personalised RT strategies, including CBCT-guided oART. However, additional factors impacting rectum motion should also be included for PTV/PRV margin computation, including patient's [293, 311, 796], treatment position [311], and changes during the treatment course [292, 294, 312, 796]. Further analyses should certainly also focus on these factors.

#### **7.4.5. Conclusions**

In this study, we evaluated the rectum motion in patients with and without rectal cancer. Using a linear mixed model, longer RT sessions were significantly correlated ( $p < 0.05$ ) with an increased intrafraction motion, regardless of tumour presence. Intra- and interfraction rectum motion were smaller in rectal cancer patients in comparison to prostate cancer patients. Interfraction motion was consistently larger than intra-fraction across all our analyses. Additionally, we observed a significant association between the rectum motion and the distance to the ano-rectal junction: the greater the

distance from the anorectal junction, the greater the rectum motion ( $p < 0.001$ ). The fixed-effect regression coefficient was higher for prostate cancer patients than for rectum cancer patients, and higher for interfraction compared to intrafraction motion. This corroborates the previous results of a greater rectum motion in prostate cancer patients compared to rectum cancer patients and, thus, suggests a fixing effect of the tumour.

These insights contribute to improve personalised pelvic RT, for which rectal motion should not be managed in the same way for prostate and rectum cancer patients. Finally, our results underscore the need for online adaptive radiotherapy development, certainly in the light of the daily interfraction variations we observed, providing short adaptive treatment sessions.

## **7.5. Appendix 5: On the trail of CBCT-guided adaptive rectal boost radiotherapy, does daily delineation require a radiation oncologist?**

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## Highlights

- The rectal boost delineation error is higher in the supero-inferior axis
- The delineation variability is lower among expert radiation oncologists
- radiotherapy technologists don't need non-expert RO oversight for rectal boost delineation in oART
- Individual training could be considered to reduce delineation variability
- AI-generated rectal boost volume is a reliable starting point during oART



## **Abstract**

### *Introduction*

Dose-escalation radiotherapy for rectal tumours is increasingly considered as a non-operative approach, with online-adaptive radiotherapy (oART) supporting this approach by correcting inter-fraction tumour position errors. However, using cone-beam computed tomography (CBCT)-guided oART requires daily target volume delineation by different operators, leading to inter-operator delineation variability and potential dosimetric issues. This study aims to compare and quantify the inter-operator and inter-professional delineation variability of the rectal boost volume on CBCT, including volumes by an automatically delineated oART treatment planning system.

### *Materials and methods*

A rectal boost volume, defined as the primary tumour extended to the entire adjacent rectal wall, was delineated on 10 CBCTs from 5 patients by 15 operators: 4 expert radiation oncologists, 4 radiation therapists, and 7 non-expert radiation oncologists. These contours were compared between the different professional groups. A comparison to the average volume of the group (radiation oncologists, radiotherapy technologists, or non-expert radiation oncologists) with the lowest delineation variability was also performed for each individual volume including the volume automatically generated by an oART treatment planning system.

## *Results*

Delineation variability was the highest in the superior (range: 2.3-6.0 mm), and inferior (2.3-12.4 mm) directions, compared to the left (0.2-4.4 mm), right (0.3-2.0 mm), anterior (0.1-2.9 mm), and posterior (0.5-4.0 mm) directions. Non-expert radiation oncologists, radiotherapy technologists, and automatic oART volume showed similar ranges of delineation errors when compared to the expert radiation oncologists' volume, which was chosen as reference volume since this professional group showed the lowest variability.

## *Discussion*

Expert radiation oncologists showed consistent results. Other professional groups exhibit similar variability, comparable to the automatic oART volume. Therefore, radiotherapy technologists could safely perform the rectal boost delineation without non-expert radiation oncologists supervision in the absence of expert radiation oncologists during CBCT-based oART. Moreover, these findings provide quantitative data to compute accurate margins for the rectal boost planning target volume in a CBCT-guided oART workflow.

### **7.5.1. Introduction**

Neoadjuvant long-course concomitant radiochemotherapy is currently recommended for treating locally advanced rectal cancer before surgery, improving local recurrence and survival rates [115, 160, 285]. This treatment paradigm is shifting towards "Total Neoadjuvant Treatment" (TNT), which combines long-course radiotherapy (RT) with chemotherapy, leading to high rates of complete responses and enabling non-operative «Watch and Wait» strategies [103, 115, 160]. However, only patients achieving a complete clinical response after this TNT are suitable for «Watch and Wait» [103, 287]. A model published by Appelt et al. 2013 indicates a RT dose-response relationship in rectal cancer, that RT dose escalation (boost) to the primary tumour could increase complete response rates. [162]. This boost can be performed using external-beam RT, either sequentially or simultaneously [103, 164, 797]. However, minimising the RT dose to the organs-at-risk, such as the small bowel, the bladder, or the non-tumorous rectum is crucial to prevent superimposed toxicities.

Cone-beam computed tomography (CBCT)-guided online-adaptive RT (oART) is emerging as a valuable technology in rectal cancer treatment. It offers the potential to reduce the planning target volume (PTV) margin by eliminating setup and interfraction anatomical variation uncertainties [194]. CBCT-guided oART incorporates an online treatment planning system (TPS), which daily delineates organs-at-risk and target volumes before generating an adapted plan. Although these delineations are initially automated, about 50% require time-consuming manual adjustments [194, 313, 314]. This

procedure is usually performed by a radiation oncologist, though radiation therapists are also integrating into these workflow for rectal RT [194]. Multiple operators, including radiation oncologists and radiotherapy technologists, may handle these delineations throughout treatment, introducing target volume delineation variability, particularly problematic when escalating doses within a larger clinical prophylactic target volume. Currently, the absence of standardised guideline for the rectal boost delineation exacerbates this variability, which could impact dosimetry significantly, potentially leading to a lack of local control due to tumour underdosage or excessive toxicities due to organ-at-risk overdosage [161, 798-800]. Additionally, the duration of the oART process is crucial, particularly in rectal radiotherapy where significant anatomical changes can occur rapidly due to physiological motion of the rectum , often within the span of several minutes, necessitating fast adaptation to ensure treatment accuracy [166]. This underscore the importance of developing workflow that reduce adaptation times.

This study aims to quantify the inter-operator and inter-professional (expert radiation oncologists, radiotherapy technologists, and non-expert radiation oncologists) delineation variability of the rectal boost volume on CBCT and to compare these with automatically delineated volumes by an oART TPS. This would provide insights for making the oART process more efficient.

## **7.5.2. Material and Methods**

### ***7.5.2.1. Sampling***

CBCT images from five patients who underwent a 5-week concurrent radiochemotherapy regimen for rectal cancer were collected and anonymized. Patients were prospectively selected based on tumour location using a convenience sampling approach to include a diversity of cases reflecting clinical practice. Children and patients with a prior history of pelvic RT were excluded. RT was performed in a supine position with a full bladder (oral water intake of 300 ml, 60 minutes before acquisition) and without rectum preparation. All patients were treated using two linear accelerators (Halcyon® and Ethos®, Varian a Siemens Healthineers Company, Palo Alto, Calif., USA). CBCTs were acquired using the “pelvis fast” (energy: 125 kV, electric charge: 560 mAs, acquisition time: 21.2 seconds) or the “pelvis large fast” (energy: 140 kV, electric charge: 672 mAs, acquisition time: 25 seconds) modes of both linear accelerators. The CBCT slice thickness was set to 2.00 mm for all the patients, with a resolution of 512 x 512 pixels (0.96 x 0.96 mm). Two CBCTs from the first RT session (one acquired before and one after RT delivery) for each patient were used to evaluate delineation uncertainties, yielding a total of 10 CBCTs. For all CBCT images, no corresponding daily magnetic resonance images (MRI) were available.

### ***7.5.2.2. Procedure and data collection***

Fifteen operators participated in this delineation study: four radiation oncologists with expertise in pelvic RT, four radiotherapy technologists, and

seven non-expert radiation oncologists. All operators were working at the same institution during the study and are actively involved in online adaptive radiotherapy delivery.

The boost clinical target volume (CTV) was defined as the rectal tumour extending to the entire adjacent rectal wall. This definition was based on a preliminary analysis assessing intra- and inter-operator reproducibility between different CTVs (see Appendix). Each of the 10 CBCTs was delineated for boost CTV by all 15 operators using the Raystation Planning system (clinical version 12A, RaySearch Laboratories, Stockholm, Sweden). To guide the delineation, the operators had access to the planning CT with the originally delineated CTV used for the treatment and a T2-weighted MRI with transversal and sagittal reconstructions obtained prior to RT planning.

In order to generate the automatic oART volume, virtual oART sessions were conducted using the oART TPS emulator of the ETHOS<sup>®</sup>, a linear accelerator dedicated to oART. This TPS integrates an artificial intelligence-based software for automatic organ-at-risk delineation. Based on the anatomy of the day of some of these organs-at-risk, called influencers, a deformable image registration is computed between the planning CT and the CBCT. Then, target volumes are propagated from the planning CT to the CBCT using the vector field from this deformable registration. In this study, the only influencer used for target volume propagation was the rectum. The automatically generated oART rectal boost volumes were collected without manual adaptation and, finally, compared to human volumes.

### 7.5.2.3. *Data analysis*

#### 7.5.2.3.1. Inter-operator and inter-professional analysis

The overall delineation variability was evaluated across all directions by generating an average volume from the rectal boost volumes delineated by all human operators. For each operator, the average deviation variability value was reported.

Additionally, the inter-professional delineation variability among expert radiation oncologists, radiotherapy technologists, and non-expert radiation oncologists was evaluated and compared. Within each professional group, the individual rectal boost volumes to the group's average volume were compared. Subsequently, the average deviation variability values were compared between each professional group. The average volume of the group (radiation oncologists, radiotherapy technologists, or non-expert radiation oncologists) with the lowest delineation variability was considered as the reference for the last part of this analysis.

Finally, the delineation variability was compared between the automatic oART volumes and the human volumes to this reference volume, thus assessing the performance and accuracy of the automated system in relation to the human-delineated volumes.

#### 7.5.2.3.2. Delineation variability analysis

The delineation variability values were acquired, for transversal (left, right, anterior, and posterior) directions, by including all the operator's volumes in the same frame of reference (Figure A5.1A) and by restricting these to the CBCT slices delineated by all the 15 operators (Figure A5.1B). Since no matched MRI was available to delineate the real contours of the tumour on the daily CBCTs, an average volume was generated from the structures created by all human operators, which was then used as the reference volume (Figure A5.1C). For the left-right direction, coronal slices were individualised from the average volume and compared them to those from each operator's delineations (Figure A5.1D). To focus only the left-right variability and eliminate the influence of antero-posterior variability, the most extreme coronal slices, representing approximately 10% of the slices, were excluded (Figure A5.1E and Figure A5.1F). For each selected slice, the left and the right distances between the average and the operator delineations at each voxel level along the supero-inferior axis were measured (Figure A5.1G). The absolute values of these distances typically followed a half-normal distribution. Therefore, the percentile 95 value of this distribution was reported for each operator and each CBCT as a measure of delineation variability (Figure A5.1H). For the antero-posterior axis, a similar analysis using sagittal slices was conducted.

To evaluate delineation variability in the supero-inferior direction, the cranial and caudal limits of the operator volumes were reported. The distance between the average of all cranial limits and the highest slice of



each operator volume was reported as the measure of delineation variability in the superior direction for each operator and each CBCT. Similarly, to determine the value in the inferior direction, the distance between the average of all caudal limits and the lowest slice of each operator volume was calculated.

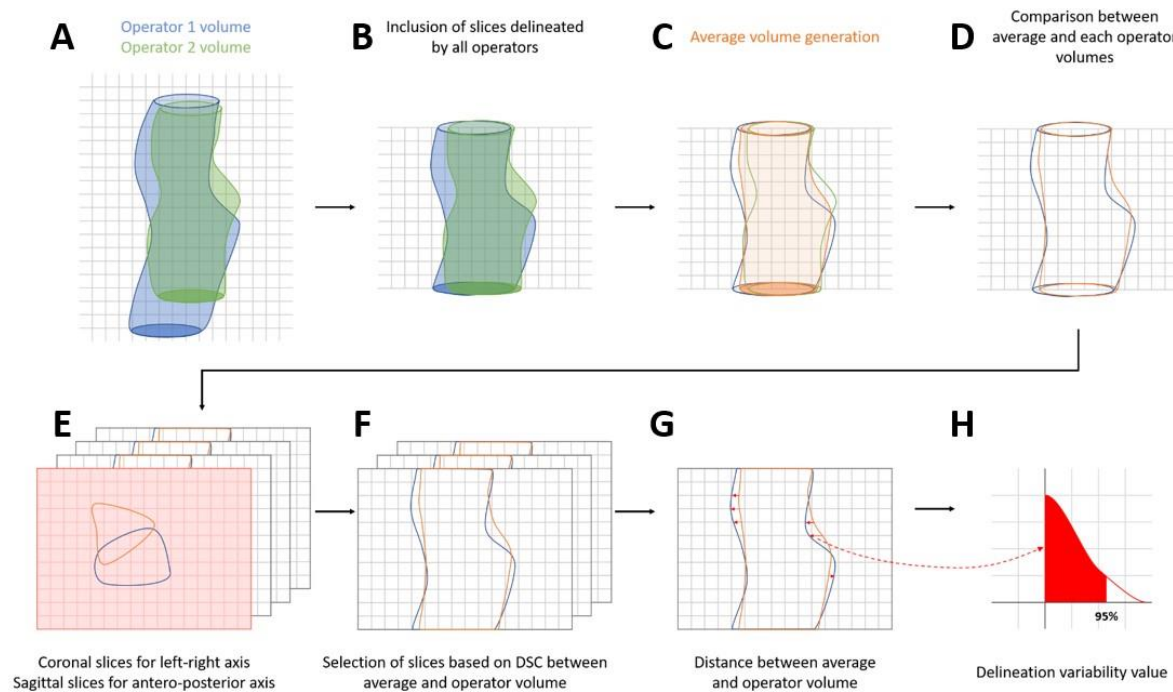


Figure A5.1: Delineation variability assessment methodology in left-right and antero-posterior directions. For a better visualisation, the volumes of 2 operators were present here, whereas the analysis was made on the volumes of 15 operators. DSC: Dice similarity coefficient.

#### 7.5.2.3.3. Statistical analysis

For the inter-operator delineation variability evaluation, the mean +/- standard deviation of the delineation variability values of each operator volume compared to the average volume was reported. These values were compared between different professional groups using a two-sided Student's t-test. A Bonferroni correction for multiple comparisons was applied when required. A p-value < 0.05 was considered statistically significant. The average volume generation and comparison with individual operators' volume were conducted using a homemade Python script (version 3.10) in Visual Studio Code (version 1.18.1). All data processing and statistical analyses were performed in RStudio (R version 4.2.1) using the "tidyverse" package.

#### 7.5.2.4. *Ethical considerations*

This monocentric study was conducted in accordance with the declaration of Helsinki and was approved by the ethics committee of the Cliniques universitaires Saint-Luc (reference number: 2022/24JUL/253).

### 7.5.3. **Results**

Patients and tumour characteristics are described in Table A5.1.

| Characteristics          | N = 5 |
|--------------------------|-------|
| <b>Gender</b>            |       |
| Male                     | 4     |
| Female                   | 1     |
| <b>Age range (years)</b> |       |
| 48 - 74                  |       |
| <b>Tumour location</b>   |       |
| Low rectum               | 2     |
| Middle rectum            | 2     |
| High rectum              | 1     |
| <b>T-stage</b>           |       |
| T3                       | 4     |
| T4                       | 1     |
| <b>Radiotherapy</b>      |       |
| Short-course             | 1     |
| Long-course              | 4     |

*Table A5.1: Demographic characteristics of the included patients*

Overall, delineation variability ranged from 0.2 to 4.4 mm on the left, 0.3 to 2.0 mm on the right, 0.1 to 2.9 mm anteriorly, 0.5 to 4.0 mm posteriorly, 2.3 to 6.0 mm superiorly, and 2.3 to 12.4 mm inferiorly among all operators (Figure A5.2).

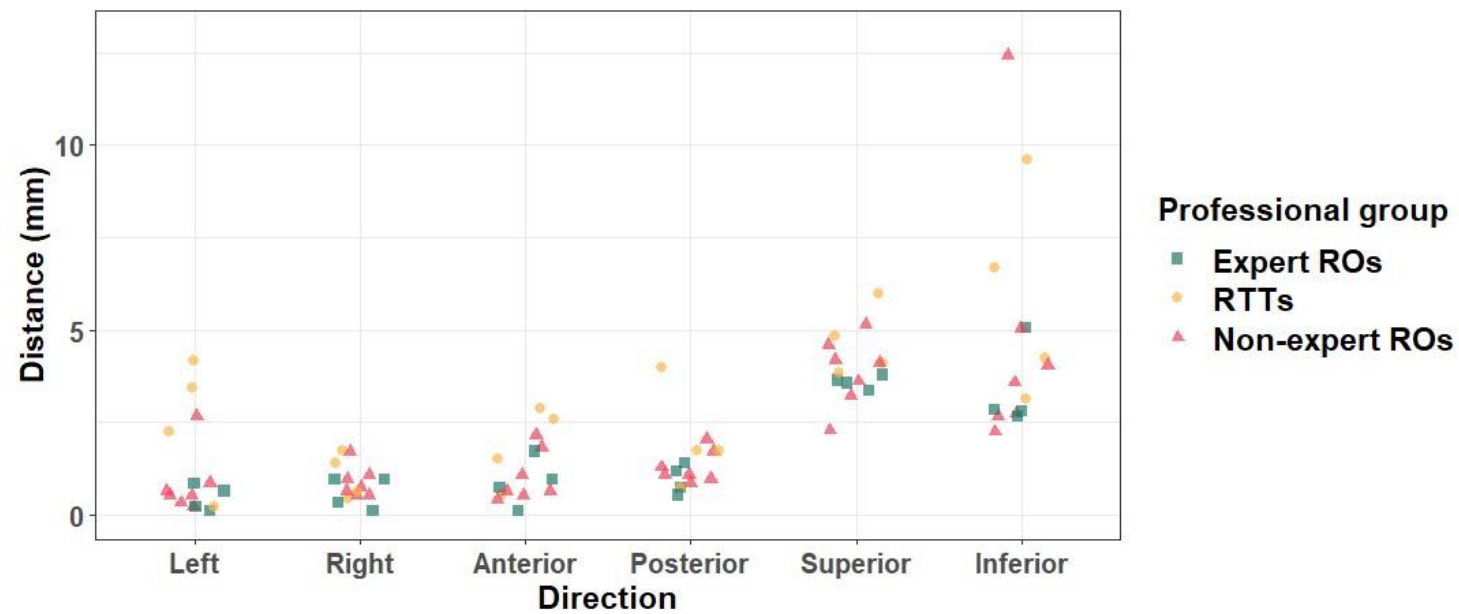


Figure A5.2: Inter-operator delineation variability in the six spatial directions compared to an average volume of all operators. ROs: Radiation oncologists, RTTs: Radiation therapists.

Inter-professional delineation variability for all directions is presented in Figure A5.3 and Table A5.2. Variability was highest among radiotherapy technologists (2.2 – 4.8 mm), followed by non-experts radiation oncologists (1.2 – 4.4 mm), and lowest among experts radiation oncologists (1.1 -3.2 mm).

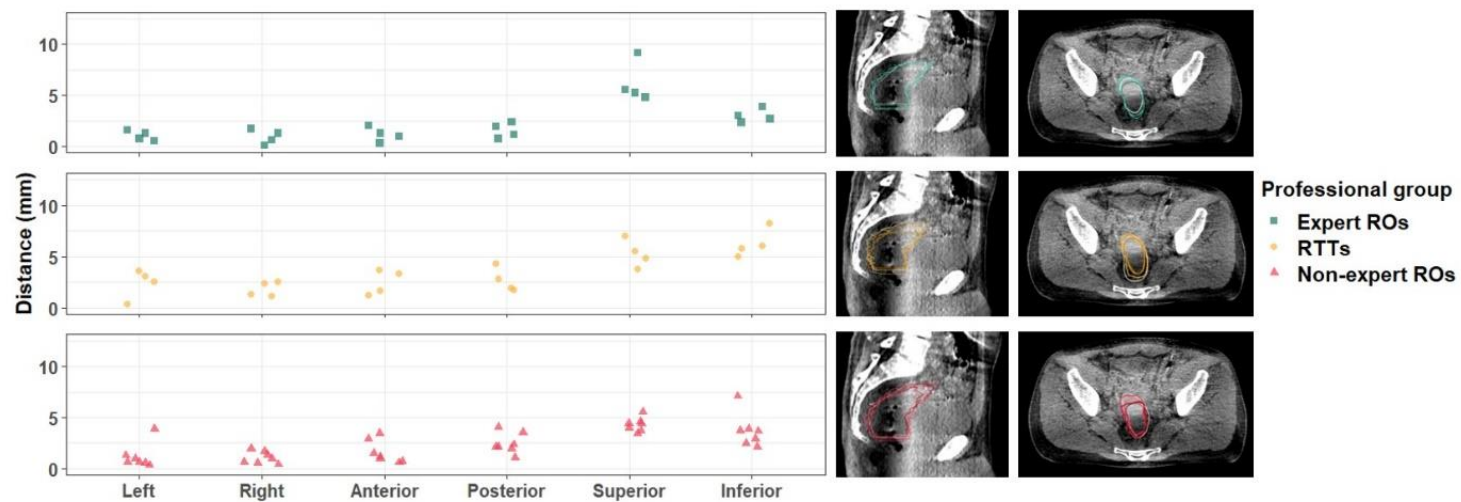


Figure A5.3: Inter-profession delineation variability in all directions. Each dot represents the mean value of the 10 rectal boost volumes delineated on CBCTs for one operator. ROs: Radiation oncologists, RTTs: Radiation therapists.

When comparing the radiotherapy technologists group to the expert radiation oncologists group, average delineation variability was significantly higher in the right ( $p < 0.001$ ), anterior ( $p = 0.002$ ), and posterior ( $p = 0.006$ ) directions. Comparing non-expert radiation oncologists and expert radiation oncologists, the delineation variability was only significantly greater in the posterior direction ( $p = 0.041$ ). Comparing non-expert radiation oncologists to radiotherapy technologists, significant difference in delineation variability was only observed in the right direction ( $p < 0.001$ ).

| Direction        | Expert radiation oncologists | radiotherapy technologists | Non-expert radiation oncologists |
|------------------|------------------------------|----------------------------|----------------------------------|
| <b>Left</b>      | 1.3 +/- 1.2                  | 4.6 +/- 8.9                | 1.4 +/-1.6                       |
| <b>Right</b>     | 1.1 +/- 1.0                  | 2.2 +/- 1.6                | 1.2 +/- 0.9                      |
| <b>Anterior</b>  | 1.3 +/- 1.3                  | 2.9 +/- 2.8                | 1.8 +/-1 .7                      |
| <b>Posterior</b> | 1.6 +/- 1.6                  | 3.1 +/- 2.3                | 2.7 +/- 2.6                      |
| <b>Superior</b>  | 3.2 +/- 2.8                  | 4.3 +/- 2.8                | 3.7 +/- 2.9                      |
| <b>Inferior</b>  | 2.9 +/- 2.3                  | 4.8 +/- 4.7                | 4.4 +/- 4.8                      |

*Table A5.2: Inter-profession delineation variability values in all directions. Each value is expressed in millimetres and is the mean of delineation errors from all volumes delineated by each operator of the professional group +/- standard deviation.*



When comparing individual contours from all groups (including automatic oART volume) with those from the expert group as reference volume, differences ranged from 0.6 to 5.0 mm on the left, 0.6 to 2.1 mm on the right, 0.8 to 3.0 mm anteriorly, 1.3 to 5.0 mm posteriorly, 2.6 to 5.8 mm superiorly, and 2.4 to 12.7 mm inferiorly (Figure A5.4). There were no significant differences between the average delineation variability of the different professional groups in any direction ( $p > 0.05$ ), nor with the automatic oART volumes ( $p > 0.05$ ).

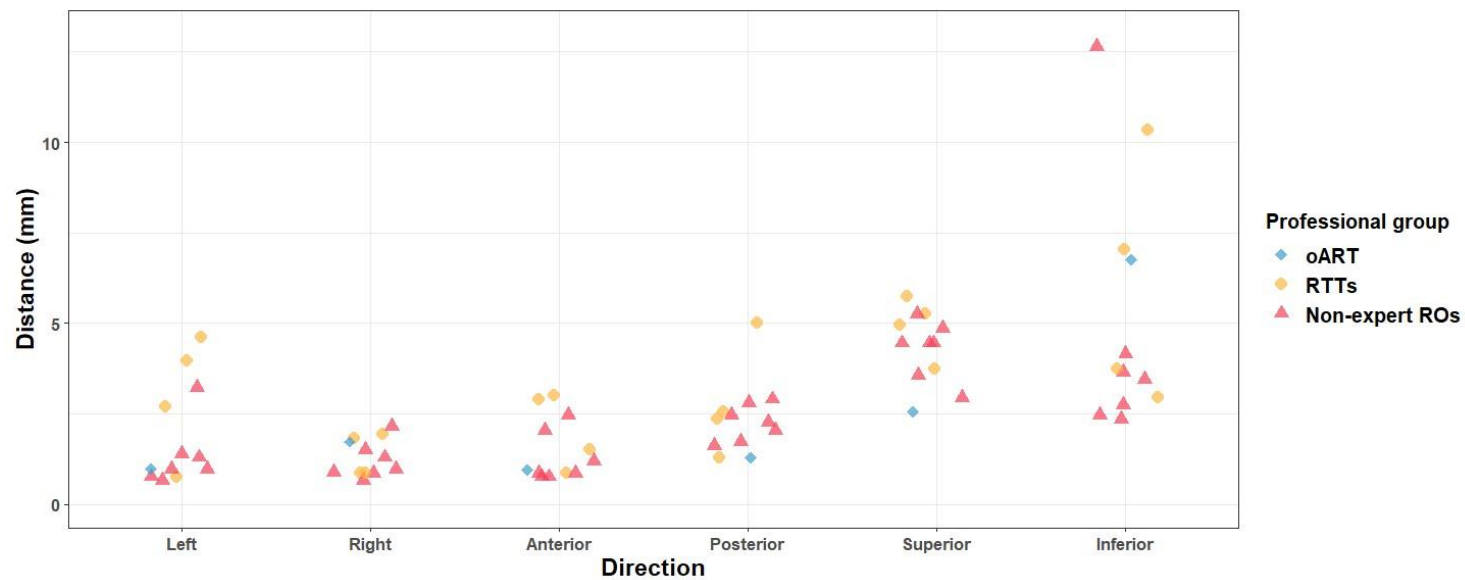


Figure A5.4: Delineation variability of automatic oART volume, radiotherapy technologists, and non-expert radiation oncologists volumes compared to the Expert radiation oncologists' average volume. oART: online-adaptive radiotherapy, ROs: Radiation oncologists, RTTs: Radiation therapists.

#### **7.5.4. Discussion**

In this study, the delineation variability of a rectal boost volume was assessed among individual operators, and different professional groups, including 4 expert radiation oncologists, 4 radiotherapy technologists, 7 non-expert radiation oncologists, and volumes automatically delineated by an oART TPS. Evaluating delineation variability on CBCTs is a first step towards performing CBCT-guided oART and incorporating this delineation error in a population-based PTV margin for rectal boost RT.

Current guidelines for rectal RT planning do not specify recommendations for boost volume, and multiple boost volume definitions are reported in literature [103, 161, 289, 290, 316, 433]. Based on preliminary analysis (see Appendix), a CTV that encompasses the entire rectal wall on transversal slices containing the tumour was adopted, as it demonstrated the lowest delineation variability. This variability has significant dosimetric implications, potentially leading to insufficient tumour control due to underdosage or increased toxicity from overdosage to organs-at-risk [798-800]. However, the designation of “CTV” could be incorrect in regard of the International Commission on Radiation Units and Measurements (ICRU) report No. 83 definition [431]. Indeed, this structure extending to the circumferential rectal wall is actually part of both the CTV (microscopic tumour infiltration) and PTV (delineation error) concepts.

Such as other reports in the literature, these analyses indicate that delineation errors were lower in transversal directions compared to supero-inferior axis [587, 589, 801], a direct consequence of the boost volume

definition. In the transversal directions, the limit was the rectal wall, which was clearly identifiable on CBCT images. In contrast, the supero-inferior boundaries, defined by the tumour's upper and lower poles, were more difficult to identify on CBCTs. Also, this greater variability is partly due to the lower resolution of the CBCT in the supero-inferior direction (2.00 mm) compared to left-right and antero-posterior directions (0.96 mm). Several strategies could be implemented to mitigate this issue and minimise delineation errors during the oART workflow. MRI-guided oART enables direct visualisation of the tumour, improving inter-operator agreement in delineating the macroscopic tumoral volume compared to CT [802]. By this way, boosting only the GTV is feasible and allows reduction of the high-dose volume compared to the broader CTV used in this study [803]. For CBCT-guided oART, implanting radio-opaque markers at the tumour's upper and lower poles during an endoscopic procedure before RT planning could serve as guide for online delineation on CBCT [167, 280]. However, MR-Linac are sparse, such as the routine implementation of radio-opaque markers. Furthermore, those radio-opaque markers could also cause artefacts that interfere with accurate MRI-based tumour response evaluation. This leaves tall the majority of the RT centers with a delineation variability that should be integrated in the PTV margins.

The contours from the radiotherapy technologist group did not significantly differ from those of the non-expert radiation oncologists group and the automatic oART ones. This is of major importance regarding the rising role of radiotherapy technologists in the new era of ART and raises questions about the necessity of further corrections by non-expert radiation

oncologists after initial adjustments by radiotherapy technologists. radiotherapy technologists are increasingly implicated in CBCT-guided oART, and workflows involving radiotherapy technologists from start to finish are already in place [194, 804, 805]. Interventions such as developing consensus guidelines, using multimodal imaging, and conducting training sessions could further reduce delineation variability [306, 587, 588]. On an individual basis, a substantial delineation variability exists inside different professional groups. For example, some radiotherapy technologists demonstrated lower variability than others when compared to the expert radiation oncologists group. This suggests that each subject could be assessed individually to determine whether the autonomy required to perform this task on a routine clinical basis has been achieved or, alternatively, whether further training is required. Furthermore, the results presented here showed that the automatic oART delineation of the boost volume is effective, providing contours comparable to those of radiotherapy technologists and non-expert radiation oncologists even without manual correction. This could eliminate the most time-consuming step in CBCT-guided oART—the manual editing of volumes—thereby enhancing workflow efficiency [313, 314]. These findings align with other studies that evaluate artificial intelligence-based segmentation of organs-at-risk and target volumes in various locations. While these volumes often still require expert manual adjustments, the time saving is significant [589, 806, 807]. In rectum oART, Ferreira Silvério et al. 2024, reported that using a deep learning model for auto-contouring in an MRI-guided workflow can provide target volumes that require no or only small adjustments in the majority of cases (70%), substantially reducing

contouring time from an average of 7 minutes 34 seconds in their conventional workflow to 3 minutes 8 seconds [589].

This study, however, had some limitations, including the small number of patients and CBCTs included and the already discussed definition of the rectal boost volume that was used. Another limitation is that the conditions under which the operators delineated the volumes did not reflect those of an actual oART session. The delineation exercise was made on a different TPS than the one dedicated to oART. Also, no time constraints were applied, although, in real clinical conditions, the RT session duration would be limited to minimise patient discomfort and significant intrafraction anatomical changes. Given the lack of daily MRI images and, consequently, the absence of a truly reliable reference volume, this study was only able to evaluate the delineation variability among several operators, but not the accuracy of their volumes compared to a groundtruth.

#### **7.5.5. Conclusions**

The delineation variability was evaluated and quantified for rectal boost volume contouring, defined as the entire rectal wall on transversal slices where the tumour is present. Inter-operator variability was more pronounced in the supero-inferior axis compared to the transversal directions (left, right, anterior, and posterior). Delineation variability was lower in the group of expert radiation oncologists. Compared to expert radiation oncologists' average volume, no significant differences in delineation variability were found between radiotherapy technologists, non-

expert radiation oncologists and a volume automatically delineated by an oART-dedicated treatment planning system. Consequently, during CBCT-guided oART, radiotherapy technologists can effectively oversee rectal boost delineation in the absence of expert radiation oncologists. However, it would be beneficial to validate this autonomy through additional training and individual assessment in order to approach the volumes of expert radiation oncologists. This study also provides essential quantitative data for the implementation of CBCT-guided rectal boost oART, including a PTV margin computation that accounts for daily delineation errors. Additionally, these findings offer deeper insights into optimising oART workflows, in which several professional groups are now in frontline.

## **7.6. Appendix 6: Investigation of changes in planning target volume and regression probability of rectal boost using in-silico cone-beam computed tomography-guided online-adaptive radiotherapy**

### **7.6.1. Tumour motion error**

We have determined a translational and a deformation part for the CTV<sub>boost</sub> motion. These two components were quantified for the inter- and intrafraction tumour motion in our cohort of 11 patients. For the interfraction motion, we compared the CTV<sub>boost</sub> delineated on planning-CT (= 'reference' volumes for interfraction motion) with the CTV<sub>boost</sub> delineated on the first CBCT of each RT sessions included in this study (= 'moving' volumes for interfraction motion). To assess the intrafraction motion, the CTV<sub>boost</sub> volume on the first CBCT of the RT session (= 'reference' volumes for intrafraction motion) was compared to CTV<sub>Boost</sub> delineated on the second and third CBCTs of the same session (= 'moving' volume for intrafraction motion). All these CTV<sub>boost</sub> volumes were delineated by the same operator on the Raystation Planning system.

For the translational motion, a rigid manual registration (without rotation) was performed between the 'reference' image and the 'moving' CBCT on the Raystation Planning system. The aim of this registration was to align the two CTV<sub>boost</sub> volumes as precisely as possible. Vector values of this registration were reported in the three axes as the translational motion error.



For the left-right deformation, we individualised coronal slices of the CTV<sub>boost</sub> volumes to be compared. We excluded the most extreme (anterior and posterior) slices to ensure that only the left-right motion was measured and to exclude the influence of an antero-posterior motion. Based on the Dice similarity coefficient ( $DSC = 2X(A \cap B)/(A+B)$ ) between the slices, we kept 90% of the slices with the greatest concordance. For each of the selected slices, we measured the left and right distances between the ‘reference’ CTV<sub>boost</sub> and the ‘moving’ CTV<sub>boost</sub> at each level along the supero-inferior axis. These distances were normally distributed with a mean equal to zero. Therefore, we reported for each moving CTV<sub>boost</sub> the standard deviation value of this distribution as the deformation motion error. For the antero-posterior deformation, we applied the same methodology using sagittal slices. For the superior and inferior directions, the motion error was the distance between, respectively, the superior and inferior slices of the reference and the moving CTV<sub>boost</sub>.

The systematic and random motion error values were computed according to van Herk et al., 2004 and reported in millimetres [489]. Accordingly, the systematic error ( $\Sigma_{TM}$ ) was the standard deviation of the distribution of the mean motion error per patient. The random error ( $\sigma_{TM}$ ) was the root mean square of the distribution of the motion error standard deviations per patient:

$$\Sigma_{TM} = \sqrt{\frac{\sum_{j=1}^N (\bar{m}_j - \bar{m})^2}{N}} \quad (Eq. A6.1)$$

and

$$\sigma_{TM} = \sqrt{\frac{\sum_{j=1}^N s_j^2}{N}} \quad (Eq. A6.2)$$

where  $N$  is the number of patients,  $\bar{m}$  is the mean of all  $\bar{m}_j$ , and  $\bar{m}_j$  is the mean of the motion error for a patient  $j$  among all RT sessions of this patient:

$$\bar{m}_j = \frac{\sum_{i=1}^n m_{i,j}}{n} \quad (Eq. A6.3)$$

where  $n$  is the number of RT sessions for a patient  $j$ , and  $m_{i,j}$  is the motion error of a session  $i$  for a patient  $j$ . The standard deviation of the motion error for a patient  $j$  ( $s_j$ ) is given by:

$$s_j = \sqrt{\frac{\sum_{i=1}^n (m_{i,j} - \bar{m}_j)^2}{n}} \quad (Eq. A6.4)$$

### 7.6.2. Delineation variability error

We have evaluated the interoperator delineation variability on 10 CBCTs from the first 5 patients enrolled in this study. CTV<sub>boost</sub> volumes were delineated on the Raystation Planning system by 15 operators. The methodology to report the delineation error values was similar to that applied for the deformation motion presented in the previous paragraph. However, for the present computation, the average volume of all operator's CTV<sub>boost</sub> was used as the 'reference' volume, and the individual CTV<sub>boost</sub>

volume of each operator was used as the ‘moving’ volume. For each operator, the distribution the distances with the average volume was expected to follow, here again, a gaussian distribution with a mean equal to zero and with a standard deviation representing the delineation error. This was made for each of the 10 CBCTs included in this analysis. The systematic ( $\Sigma_D$ ) and random ( $\sigma_D$ ) delineation error values were computed as follow:

$$\Sigma_D = \sqrt{\frac{\sum_{k=1}^O d_k^2}{O}} \text{ (Eq. A6.5)}$$

and

$$\sigma_D = \sqrt{\frac{\sum_{k=1}^O \delta_k^2}{O}} \text{ (Eq. A6.6)}$$

the number of operators is  $O$ ,  $d_k$  and  $\delta_k$  refers to the systematic and random delineation error for an operator  $k$  given by:

$$d_k = \sqrt{\frac{\sum_{j=1}^N (\bar{d}_{j,k} - \bar{d}_k)^2}{N}} \text{ (Eq. A6.7)}$$

$$\delta_k = \sqrt{\frac{\sum_{j=1}^N \delta_{j,k}^2}{N}} \text{ (Eq. A6.8)}$$

respectively, where  $N$  is the number of patients,  $\bar{d}_k$  is the mean of all  $\bar{d}_{j,k}$ , and  $\bar{d}_{j,k}$  is the mean of the delineation error for a patient  $j$  delineated by an operator  $k$  among all RT sessions of this patient:

$$\bar{d}_{j,k} = \frac{\sum_{i=1}^n d_{i,j,k}}{n} \text{ (Eq. A6.9)}$$

where  $n$  is the number of RT sessions for a patient  $j$ , and  $d_{i,j,k}$  is the delineation error of a session  $i$  for a patient  $j$  delineated by an operator  $k$ .

The standard deviation of the delineation error for a patient  $j$  delineated by an operator  $k$  ( $\delta_{j,k}$ ) is given by :

$$\delta_{j,k} = \sqrt{\frac{\sum_{i=1}^n (d_{i,j,k} - \bar{d}_{j,k})^2}{n}} \text{ (Eq. A6.10)}$$

### 7.6.3. Setup error and penumbra

The values of setup errors were extracted from the literature and adapted to our clinical protocols [295].

The penumbra value was determined according to the method described by Wanet et al. and adapted to the linear accelerator and treatment planning system specificities [173, 301].

## 7.7. Appendix 7: Radiotherapy boost to the primary tumour in locally advanced rectal cancer: Systematic review of practices and Meta-analysis

### 7.7.1. Conversion of radiotherapy doses in biological equivalent doses.

The conventional linear quadratic model was used to obtain the biologically effective doses (BED) for each study [420]:

$$BED = n \times d \times \left( 1 + \frac{d}{\alpha/\beta} \right) \text{ Eq. A7.1}$$

Where  $n$  is the number of RT sessions,  $d$  is the dose per session, and  $\alpha/\beta$  is the ratio between the linear and quadratic components of cell killing. In case of using bifractionated RT or a varying dose between different RT sessions, the modified linear quadratic model incorporating time factor published by van de Geijn et al., 1989 [458] was used:

$$BED = n \times d \times \left( 1 + \frac{d}{\alpha/\beta} \right) - \frac{\ln(2) \times (t_n - t_k)}{(\alpha \times t_p)} \text{ Eq. A7.2}$$

Where  $t_n$  is the total RT duration,  $t_k$  is the time to repopulation, and  $t_p$  is the doubling time for proliferation of rectal cancer. Values for rectal cancer were retrieved from the literature and set to  $\alpha/\beta = 5.06$  Gy [320],  $\alpha = 0.339$  Gy [320],  $t_k = 21$  days [808], and  $t_p = 5.9$  days [809].

The Table A7.1 shows the Biologically effective dose conversion of RT doses frequently used in clinical practice

| N fractions                                                    | Dose per fraction (Gy)                | Total BED (Gy) |
|----------------------------------------------------------------|---------------------------------------|----------------|
| 5                                                              | 5                                     | 49.7           |
| 25                                                             | 1.8                                   | 61.0           |
| 25                                                             | 2                                     | 69.8           |
| 25                                                             | 2.2                                   | 78.9           |
| 28                                                             | 1.8                                   | 68.3           |
| 30                                                             | 1.8                                   | 73.2           |
| 25                                                             | 1.96                                  | 68.0           |
| 25                                                             | 2.09                                  | 74.0           |
| 50 (BID)                                                       | 1.2                                   | 69.4           |
| 25 + 5 second daily<br>fractions the last week<br>of treatment | 1.8 (+ second daily<br>fraction: 1.5) | 76.2           |
| 24.4                                                           | 2                                     | 68.0           |
| 26.5                                                           | 2                                     | 74.0           |

*Table A7.1: Conversion table of frequently used radiotherapy doses in BED. BED: Biologically effective dose, BID: Bis in die (Twice daily).*

### **7.7.2. Results of the meta-analysis for clinical complete response in «Watch and Wait» studies and local recurrence rate in planned and «Watch and Wait» studies.**

#### **7.7.2.1. Clinical complete response results in «Watch and Wait» studies**

In studies evaluating «Watch and Wait» strategy, there was no significant impact of the RT technique ( $p = 0.24$ ), the CTV definition ( $p = 0.69$ ), the PTV definition ( $p = 0.19$ ), the boost sequence ( $p = 0.07$ ), the boost dose ( $p = 0.25$ ),

the concomitant chemotherapy ( $p = 0.36$ ), and the induction/consolidation chemotherapy ( $p = 0.54$ ) on the clinical complete response rate. The publication year was not evaluated here since all included «Watch and Wait» studies were published after 2016 (Figure A7.1 and Figure A7.7 for detailed results).

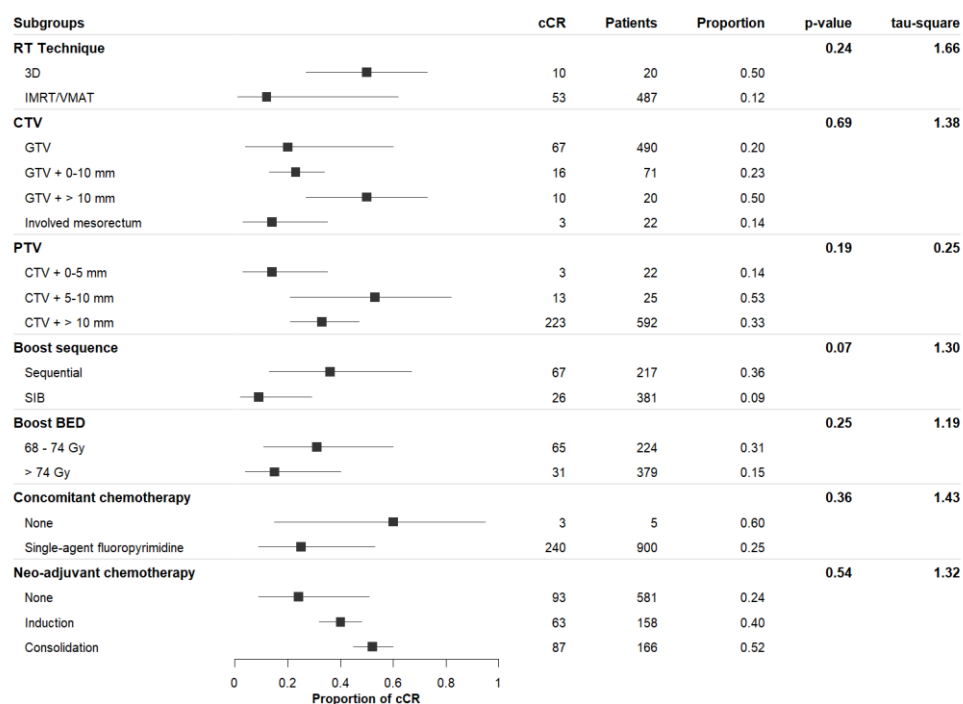


Figure A7.1: Subgroup meta-analysis of «Watch and Wait» studies reporting clinical complete response rate. The “cCR” column is the number of clinical complete response events within subgroup, the “Patients” column reported the total of the patients within each subgroup among the pooled publications, the “Proportion” column is the estimated clinical complete response rate within each subgroup, the “p-value” column is the p-value for subgroup differences analysed by a mixed-effects model using the Q-test, and the “tau-square” column refers to the between-study heterogeneity variance assessed using the maximum likelihood estimator. 3D: Three-

dimensional radiotherapy, BED: Biologically effective dose, cCR: Clinical complete response, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

The heterogeneity among studies quantified by the  $I^2$  statistics was important (range: 82 - 94%,  $p < 0.01$ ) for all the parameters analysed (Table A7.2).

| $I^2$  | $\tau^2$ | Q test of heterogeneity<br>p-value |
|--------|----------|------------------------------------|
| < 25%  | <0.1     |                                    |
| < 50%  | <0.3     | >0.05                              |
| < 75%  | <1       | <0.05                              |
| < 100% | >1       | <0.01                              |

| Meta-analysis of clinical complete response<br>in «Watch and Wait» publications | $I^2$ | $\tau^2$ | Q test of heterogeneity<br>p-value |
|---------------------------------------------------------------------------------|-------|----------|------------------------------------|
| <b>RT Modulation</b>                                                            | 94%   | 1.66     | < 0.01                             |
| 3D                                                                              |       |          | NA                                 |
| IMRT/VMAT                                                                       | 94%   | 0.95     | < 0.01                             |
| <b>CTV</b>                                                                      | 91%   | 1.38     | < 0.01                             |
| GTV                                                                             | 95%   | 1.07     | < 0.01                             |
| GTV + 0-10 mm                                                                   |       |          | NA                                 |
| GTV + > 10 mm                                                                   |       |          | NA                                 |
| Involved mesorectum                                                             |       |          | NA                                 |
| <b>PTV</b>                                                                      | 82%   | 0.25     | < 0.01                             |
| CTV + 0-5 mm                                                                    |       |          | NA                                 |
| CTV + 5-10 mm                                                                   | 0%    | 0.15     | 0.69                               |
| CTV + > 10 mm                                                                   | 89%   | 0.15     | < 0.01                             |
| <b>Boost sequence</b>                                                           | 92%   | 1.30     | < 0.01                             |
| Sequential                                                                      | 77%   | 0.58     | 0.01                               |
| SIB                                                                             | 93%   | 0.58     | < 0.01                             |
| <b>Boost BED</b>                                                                | 89%   | 1.19     | < 0.01                             |
| 68 - 74 Gy                                                                      | 75%   | 0.90     | < 0.01                             |



|                                  |     |      |        |
|----------------------------------|-----|------|--------|
| > 74 Gy                          | 92% | 0.90 | < 0.01 |
| <b>Concomitant chemotherapy</b>  | 94% | 1.43 | < 0.01 |
| None                             | NA  |      |        |
| Single-agent fluoropyrimidine    | 95% | 1.28 | < 0.01 |
| <b>Neo-adjuvant chemotherapy</b> | 93% | 1.32 | < 0.01 |
| None                             | 92% | 1.06 | < 0.01 |
| Induction                        | NA  |      |        |
| Consolidation                    | NA  |      |        |

*Table A7.2: Heterogeneity analysis of the clinical complete response meta-analysis of «Watch and Wait» publications. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, NA: Not applicable, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

No significant publication bias was highlighted (Peters' test p-value = 0.90, Figure A7.2).

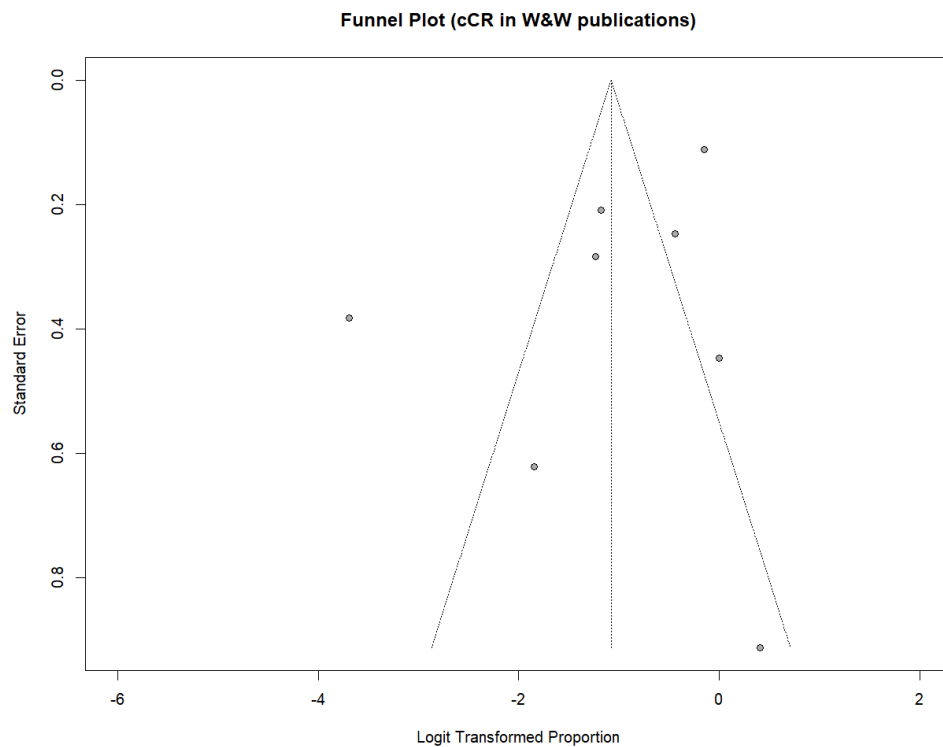


*Figure A7.2: Funnel plot of «Watch and Wait» publications reporting clinical complete response. cCR: Clinical complete response, W&W: Watch and Wait.*

#### **7.7.2.2. Local recurrence rate results in planned surgery studies**

In studies with planned surgery, there was no significant impact of the publication year ( $p = 0.18$ ), the RT technique ( $p = 0.53$ ), the CTV definition ( $p = 0.38$ ), the PTV definition ( $p = 0.29$ ), the boost sequence ( $p = 0.57$ ), the boost dose ( $p = 0.88$ ), the concomitant chemotherapy ( $p = 0.36$ ), the induction/consolidation chemotherapy ( $p = 0.52$ ), and the surgical delay ( $p = 0.98$ ) on the local recurrence rate rate (Figure A7.3 and Figure A7.8 for detailed results).

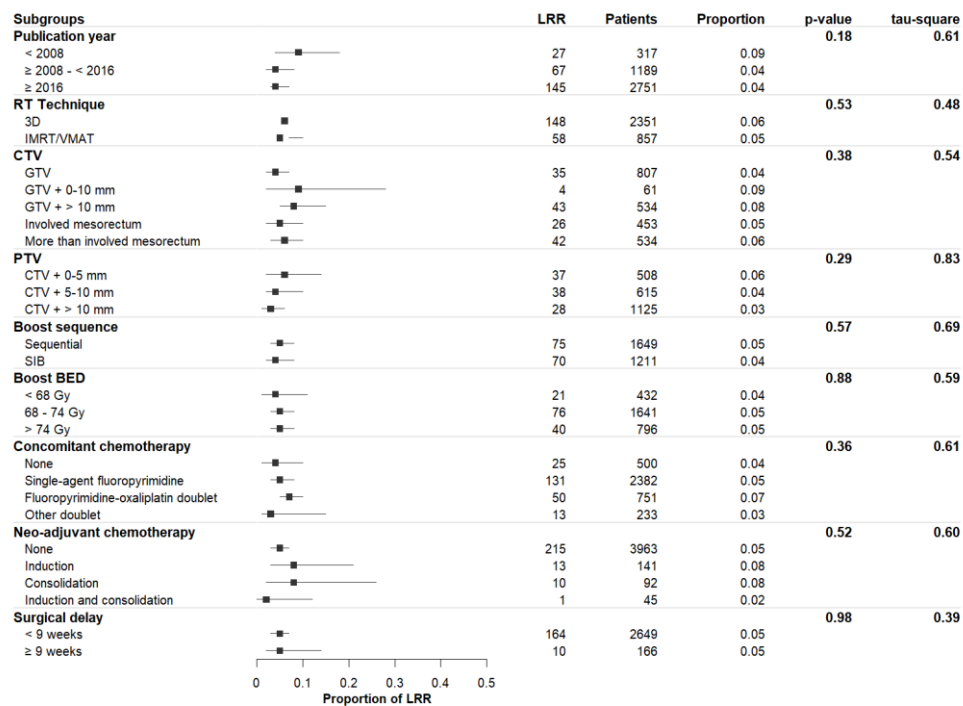


Figure A7.3: Subgroup meta-analysis of planned surgery studies reporting local recurrence rate rate. The “LRR” column is the number of local recurrence rate events within subgroup, the “Patients” column reported the total of the patients within each subgroup among the pooled publications, the “Proportion” column is the estimated local recurrence rate rate within each subgroup, the “p-value” column is the p-value for subgroup differences analysed by a mixed-effects model using the Q-test, and the “tau-square” column refers to the between-study heterogeneity variance assessed using the maximum likelihood estimator. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, LRR: Local relapse rate, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

The heterogeneity among studies quantified by the  $I^2$  statistics was moderate (range: 12 - 59%,  $p$ : < 0.01 – 0.28) for all the parameters analysed (Table A7.3).

| Meta-analysis of local recurrence rate in planned surgery publications | $I^2$ | $\tau^2$ | Q test of heterogeneity p-value |
|------------------------------------------------------------------------|-------|----------|---------------------------------|
| <b>Publication year</b>                                                | 52%   | 0.61     | < 0.01                          |
| < 2008                                                                 | 45%   | 0.60     | 0.08                            |
| ≥ 2008 - < 2016                                                        | 49%   | 0.76     | 0.02                            |
| ≥ 2016                                                                 | 53%   | 0.42     | < 0.01                          |
| <b>RT Modulation</b>                                                   | 30%   | 0.48     | 0.06                            |
| 3D                                                                     | 0%    | 0.00     | 0.83                            |
| IMRT/VMAT                                                              | 43%   | 1.31     | 0.03                            |
| <b>CTV</b>                                                             | 36%   | 0.54     | 0.02                            |
| GTV                                                                    | 41%   | 0.38     | 0.09                            |
| GTV + 0-10 mm                                                          | 0%    | 0.38     | 0.99                            |
| GTV + > 10 mm                                                          | 38%   | 0.38     | 0.15                            |
| Involved mesorectum                                                    | 0%    | 0.38     | 0.87                            |
| More than involved mesorectum                                          | 0%    | 0.38     | 0.75                            |
| <b>PTV</b>                                                             | 59%   | 0.83     | < 0.01                          |
| CTV + 0-5 mm                                                           | 35%   | 0.65     | 0.15                            |
| CTV + 5-10 mm                                                          | 22%   | 1.40     | 0.22                            |
| CTV + > 10 mm                                                          | 56%   | 0.23     | 0.03                            |
| <b>Boost sequence</b>                                                  | 55%   | 0.69     | < 0.01                          |
| Sequential                                                             | 60%   | 0.44     | < 0.01                          |
| SIB                                                                    | 49%   | 1.13     | < 0.01                          |
| <b>Boost BED</b>                                                       | 48%   | 0.59     | < 0.01                          |
| < 68 Gy                                                                | 0%    | 0.22     | 0.59                            |
| 68 - 74 Gy                                                             | 61%   | 0.59     | < 0.01                          |
| > 74 Gy                                                                | 44%   | 0.76     | 0.03                            |
| <b>Concomitant chemotherapy</b>                                        | 52%   | 0.61     | < 0.01                          |
| None                                                                   | 55%   | 0.44     | 0.05                            |

|                                      |     |      |        |
|--------------------------------------|-----|------|--------|
| Single-agent fluoropyrimidine        | 61% | 0.67 | < 0.01 |
| Fluoropyrimidine-oxaliplatin doublet | 21% | 0.00 | 0.28   |
| Other doublet                        | 25% | 1.29 | 0.24   |
| <b>Neo-adjuvant chemotherapy</b>     | 53% | 0.60 | < 0.01 |
| None                                 | 52% | 0.52 | < 0.01 |
| Induction                            | 67% | 0.52 | 0.05   |
| Consolidation                        | 0%  | 0.52 | 0.99   |
| Induction and consolidation          | NA  |      |        |
| <b>Surgical delay</b>                | 12% | 0.39 | 0.28   |
| < 9 weeks                            | 17% | 0.39 | 0.23   |
| ≥ 9 weeks                            | 0%  | 0.39 | 0.95   |

*Table A7.3: Heterogeneity analysis of the local recurrence rate meta-analysis of planned surgery publications. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, NA: Not applicable, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

Additionally, a significant publication bias was highlighted (Peters' test p-value = 0.005, Figure A7.4).

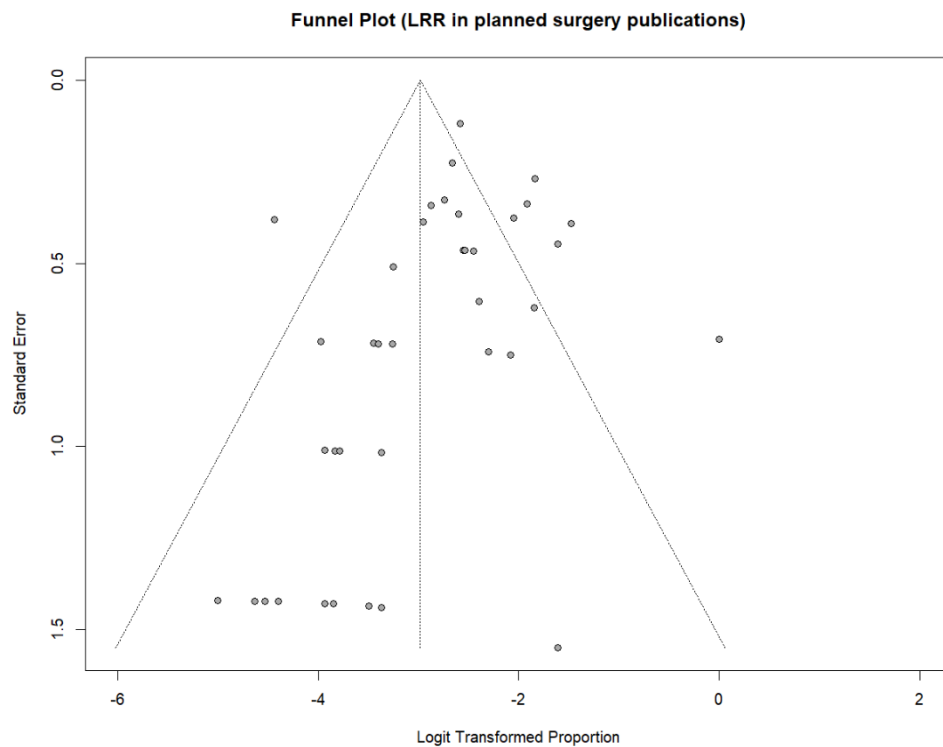


Figure A7.4: Funnel plot of planned surgery publications reporting local recurrence rate. LRR: Local recurrence rate.

### 7.7.2.3. Local recurrence rate results in «Watch and Wait» studies

In «Watch and Wait» studies, there was no significant impact of the RT technique ( $p = 0.24$ ), the PTV definition ( $p = 0.97$ ), the boost sequence ( $p = 0.66$ ), the boost dose ( $p = 0.33$ ), and the concomitant chemotherapy ( $p = 0.95$ ). However, an exception was noted: the use of induction or consolidation chemotherapy was associated with a higher local recurrence rate ( $p = 0.015$ ), which is contra-intuitive. The publication year and the CTV

definition were not evaluated here since all studies are in the same category (publication year  $\geq 2016$ , CTV defined as the GTV without margin, Figure A7.5 and Figure A7.9 for detailed results).

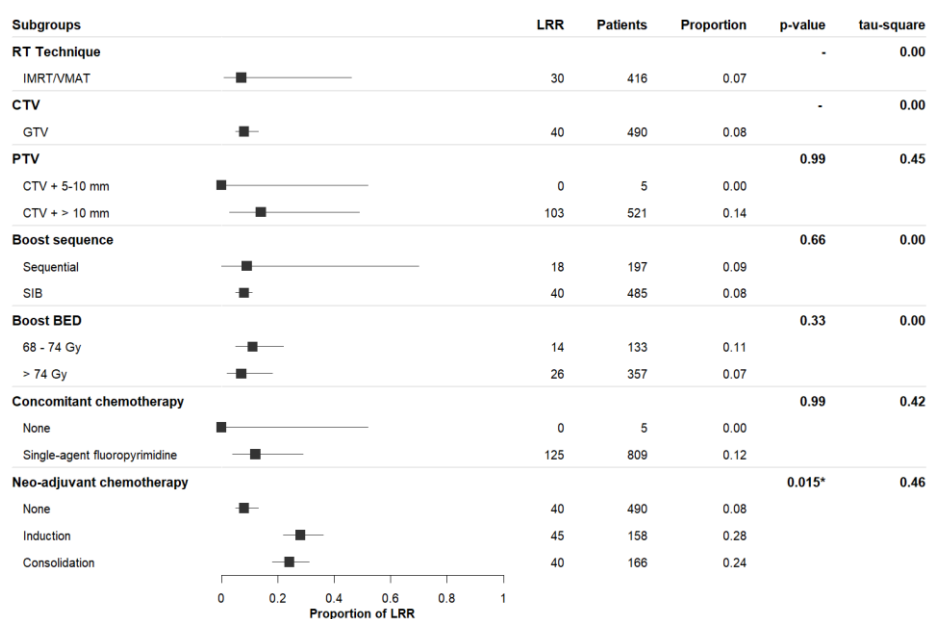


Figure A7.5: Subgroup meta-analysis of «Watch and Wait» reporting local recurrence rate. The “LRR” column is the number of local recurrence rate events within subgroup, the “Patients” column reported the total of the patients within each subgroup among the pooled publications, the “Proportion” column is the estimated local recurrence rate within each subgroup, the “p-value” column is the p-value for subgroup differences analysed by a mixed-effects model using the Q-test, and the “tau-square” column refers to the between-study heterogeneity variance assessed using the maximum likelihood estimator. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, LRR: Local relapse rate, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

The heterogeneity among studies quantified by the  $I^2$  statistics varied widely between the parameters evaluated (range: 5 - 94%,  $p$ : < 0.01 – 0.38, Table A7.4).

| Meta-analysis of local recurrence rate in «Watch and Wait» publications | $I^2$ | $\tau^2$ | Q test of heterogeneity p-value |
|-------------------------------------------------------------------------|-------|----------|---------------------------------|
| <b>RT Modulation (= IMRT/VMAT)</b>                                      | 0%    | 0.00     | 0.61                            |
| <b>CTV (= GTV)</b>                                                      | 28%   | 0.00     | 0.24                            |
| <b>PTV</b>                                                              | 86%   | 0.45     | < 0.01                          |
| CTV + 5-10 mm                                                           | NA    |          |                                 |
| CTV + > 10 mm                                                           | 91%   | 0.43     | < 0.01                          |
| <b>Boost sequence</b>                                                   | 52%   | 0.00     | 0.12                            |
| Sequential                                                              | 71%   | 0.00     | 0.06                            |
| SIB                                                                     | NA    |          |                                 |
| <b>Boost BED</b>                                                        | 5%    | 0.00     | 0.38                            |
| 68 - 74 Gy                                                              | 56%   | 0.00     | 0.13                            |
| > 74 Gy                                                                 | 0%    | 0.00     | 0.93                            |
| <b>Concomitant chemotherapy</b>                                         | 91%   | 0.42     | < 0.01                          |
| None                                                                    | NA    |          |                                 |
| Single-agent fluoropyrimidine                                           | 93%   | 0.42     | < 0.01                          |
| <b>Neo-adjuvant chemotherapy</b>                                        | 89%   | 0.46     | < 0.01                          |
| None                                                                    | 28%   | 0.00     | 0.24                            |
| Induction                                                               | NA    |          |                                 |
| Consolidation                                                           | NA    |          |                                 |

Table A7.4: Heterogeneity analysis of the local recurrence rate meta-analysis of «Watch and Wait» publications. BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, NA: Not applicable, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.



No significant publication bias was highlighted (Peters' test p-value = 0.64, Figure A7.6).

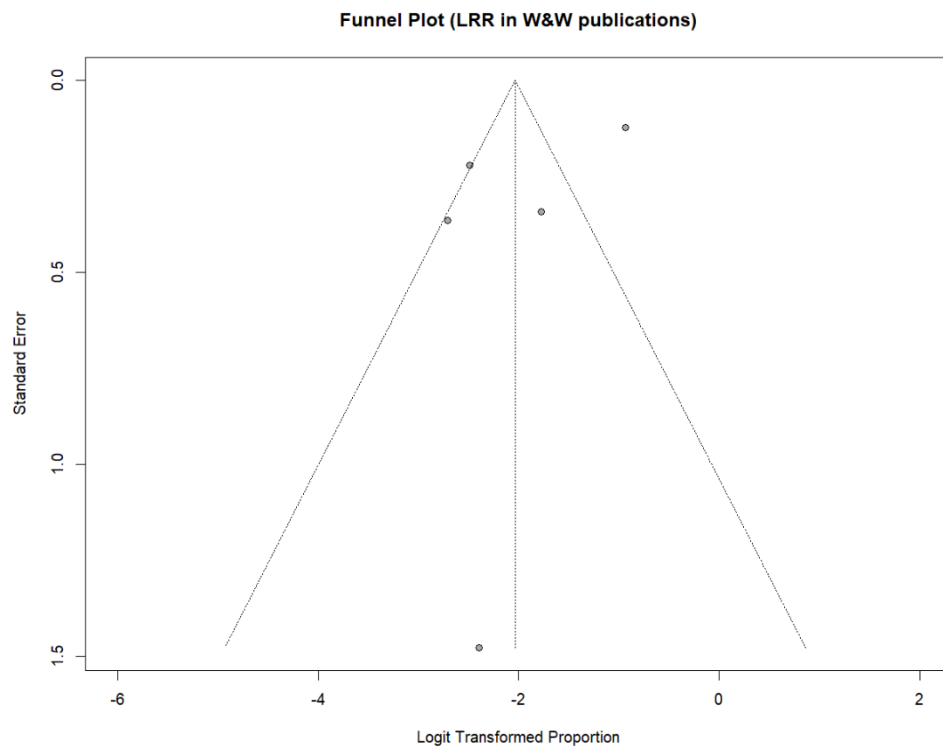
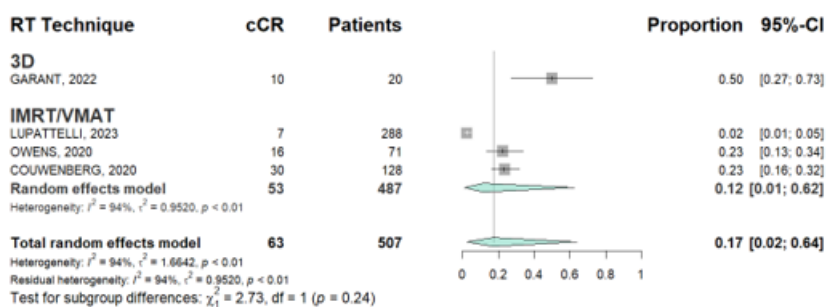


Figure A7.6: Funnel plot of «Watch and Wait» publications reporting local recurrence rate. LRR: Local recurrence rate, W&W: Watch and Wait.

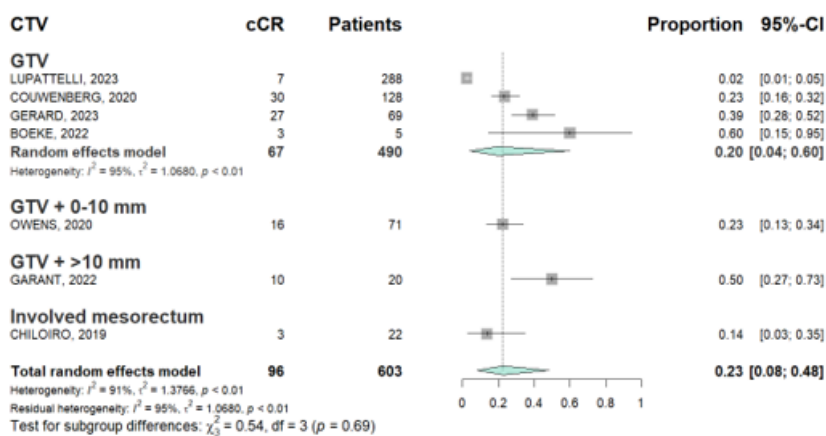
#### 7.7.2.4. Detailed meta-analysis results

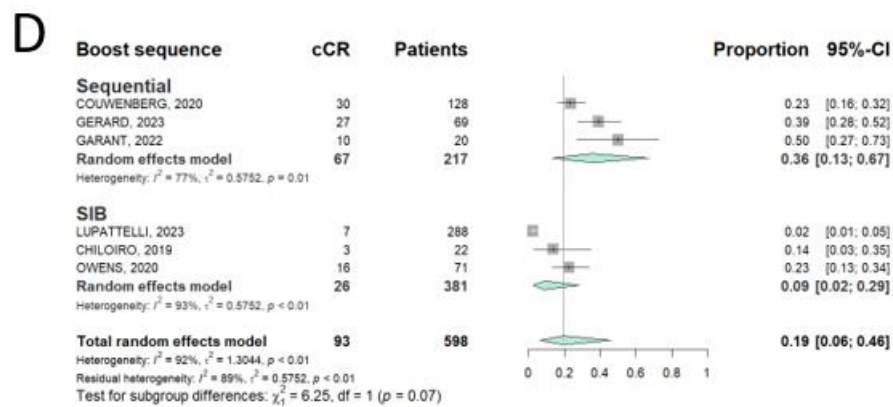
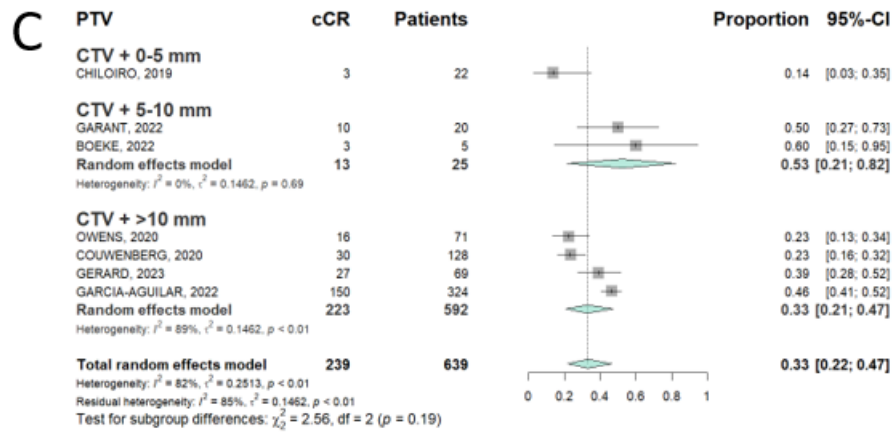
##### 7.7.2.4.1. Clinical complete response results in «Watch and Wait» studies

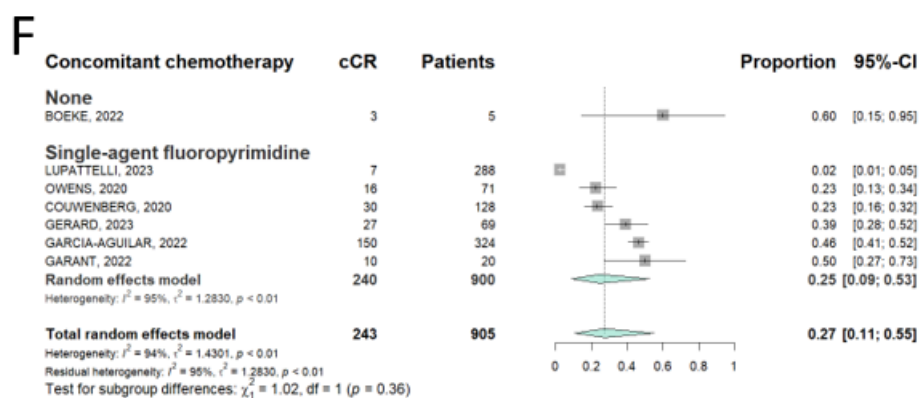
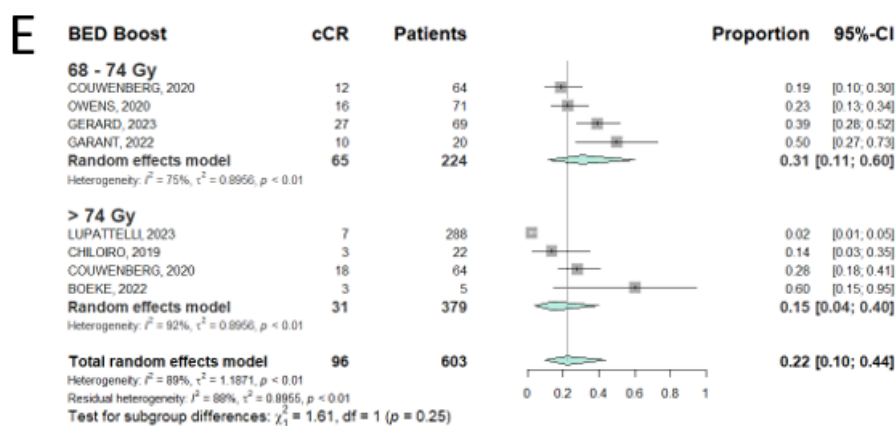
A



B







G

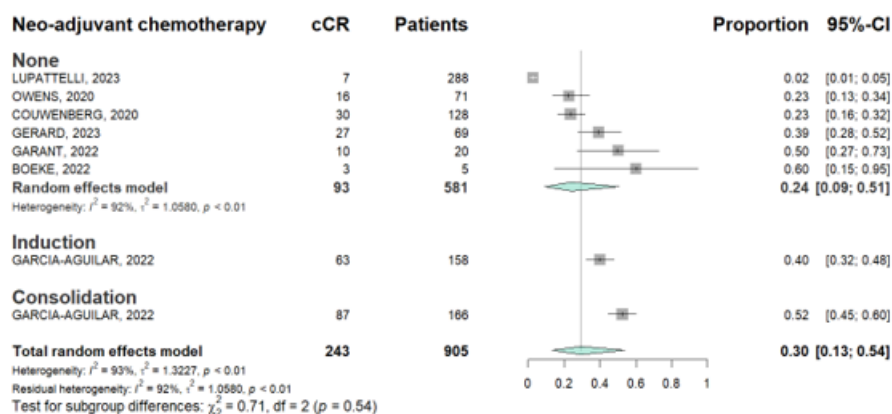
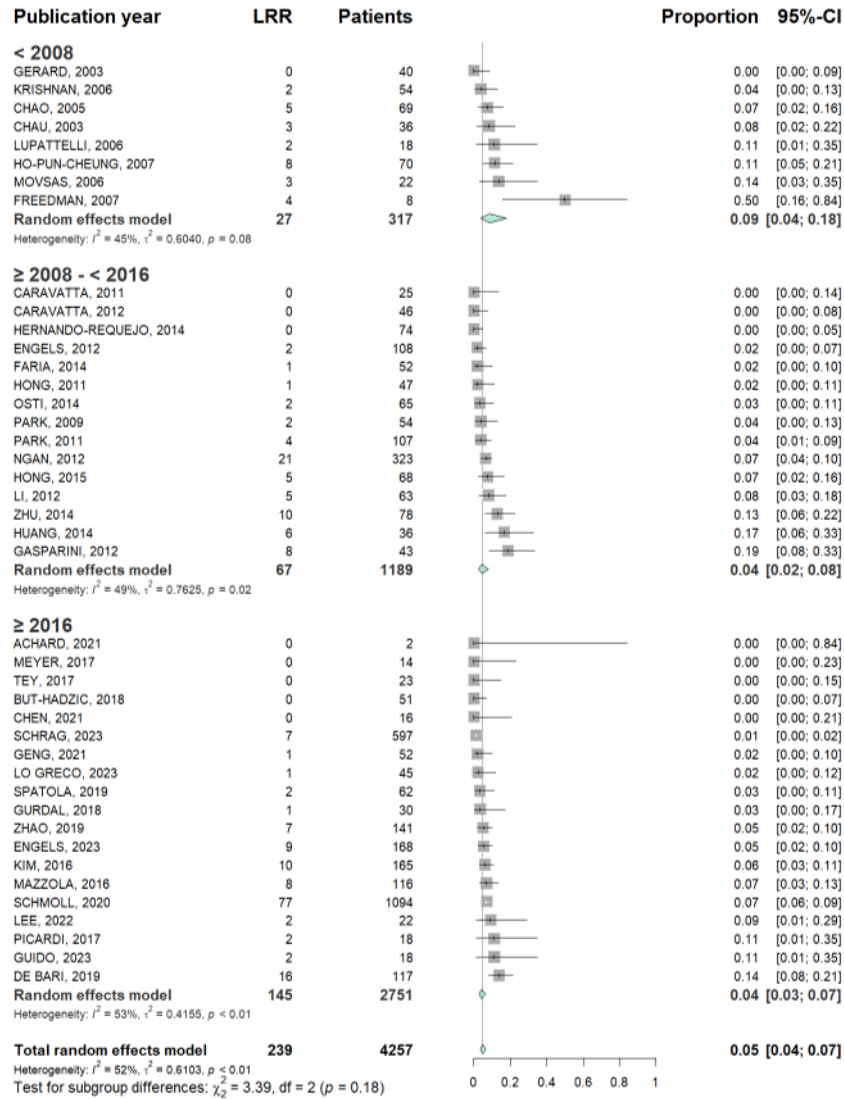


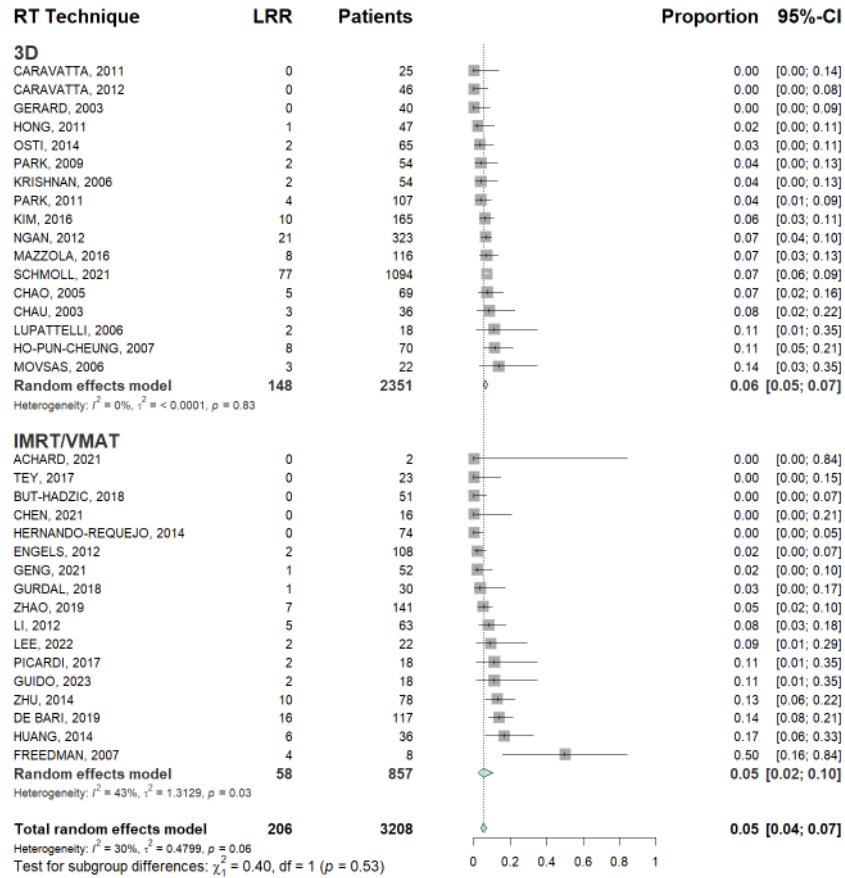
Figure A7.7: Details of the meta-analysis of «Watch and Wait» studies reporting clinical complete response rate as function of (A) RT modulation, (B) CTV definition, (C) PTV definition, (D) boost sequence, (E) boost BED, (F) concomitant chemotherapy, and (G) neo-adjuvant chemotherapy. The “cCR” column is the number of clinical complete response events within each study, the “Patients” column reported the total of the patients within each study among the pooled publications, the “Proportion” column is the clinical complete response rate within each study, the “95%-CI” column is the 95% confidence interval of the local recurrence rate rate within each study. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, cCR: Clinical complete response, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

#### 7.7.2.4.2. Local recurrence rate results in planned surgery studies

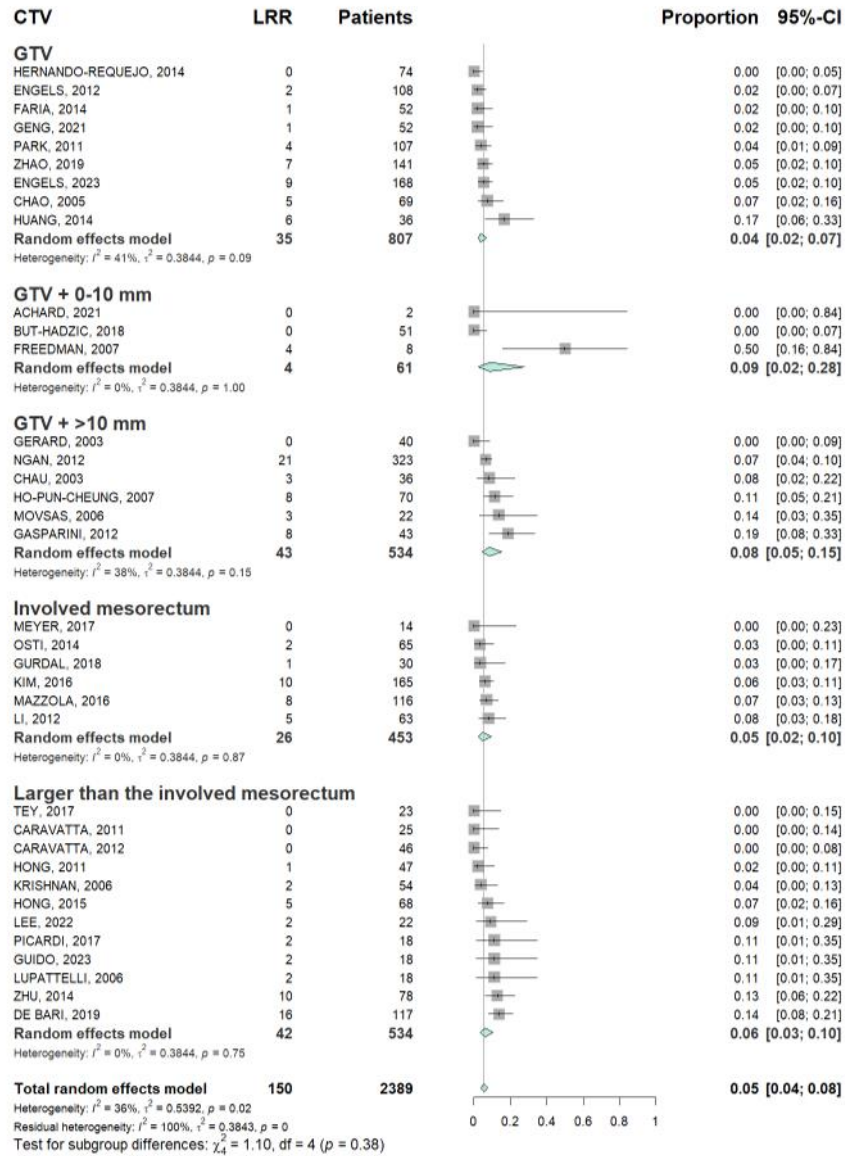
A



B

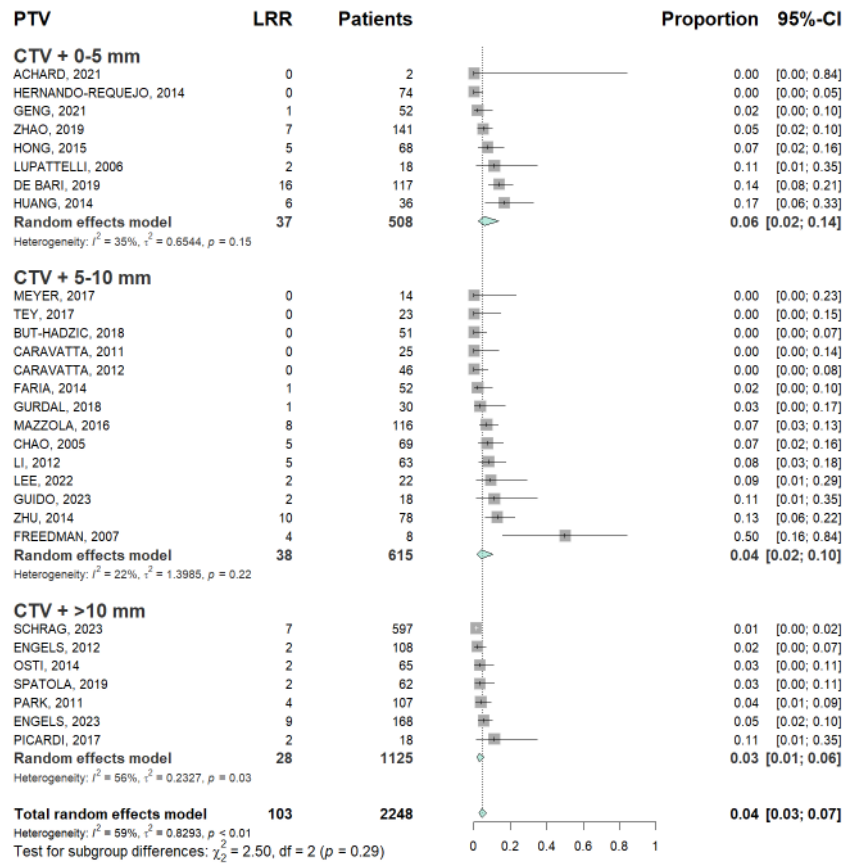


C

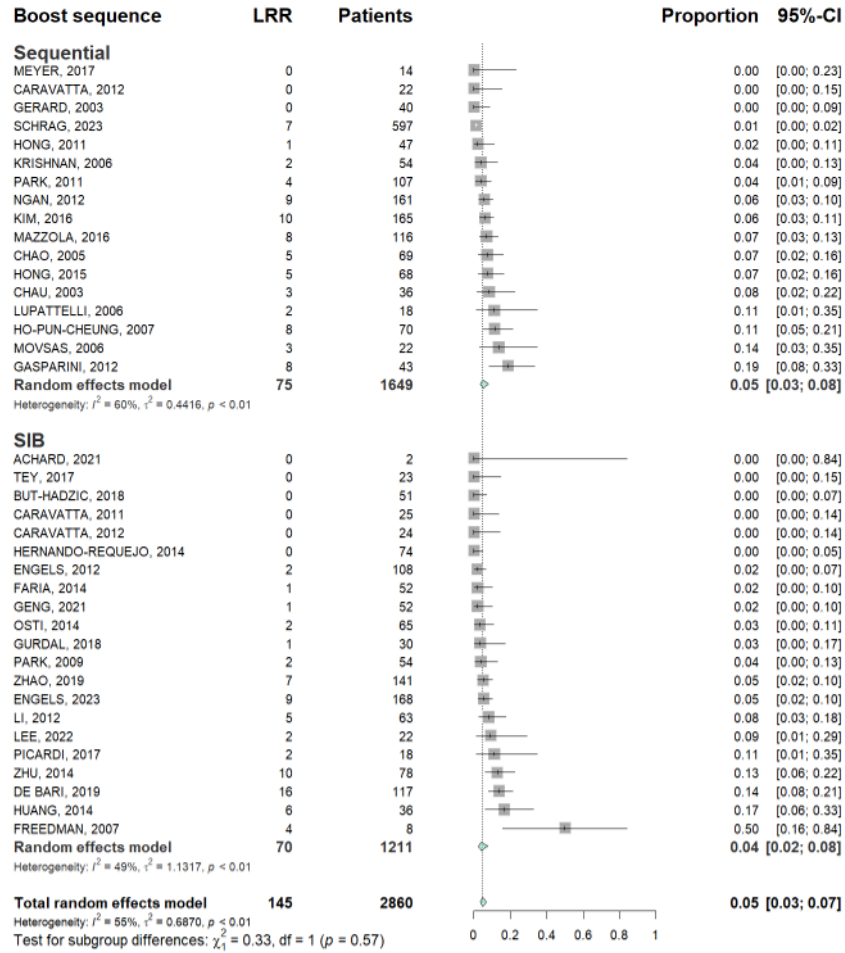




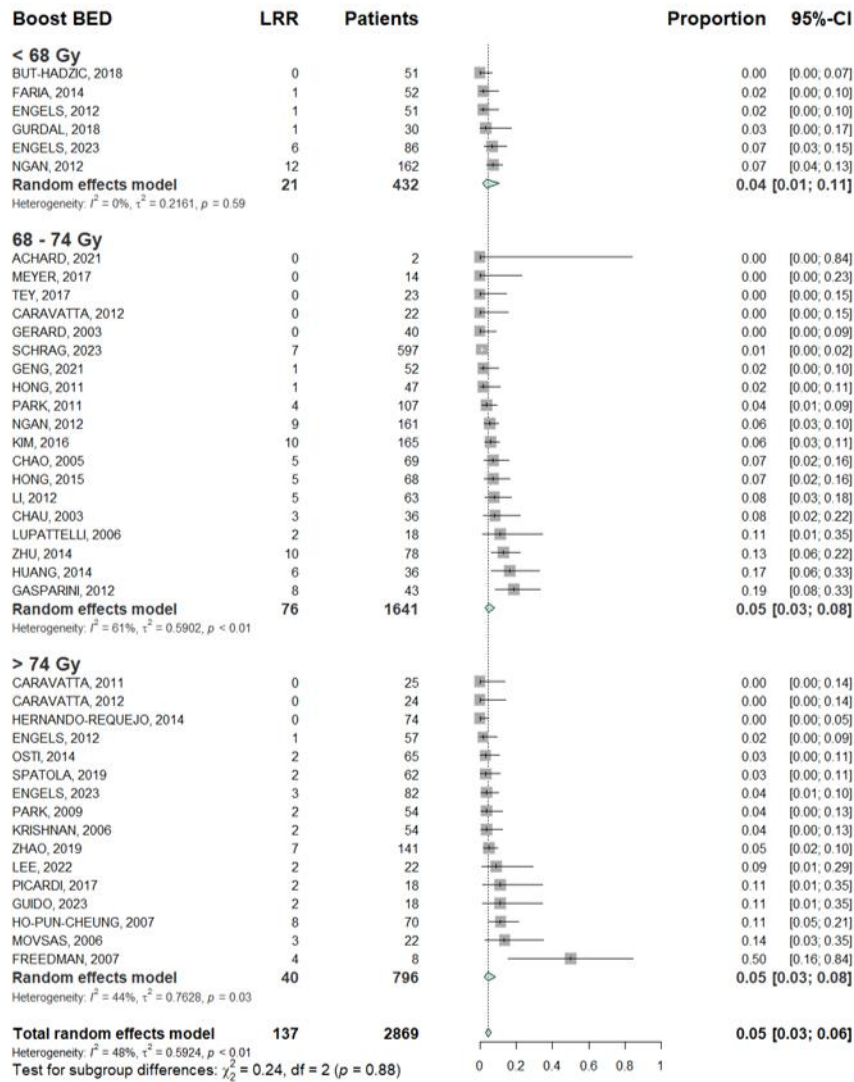
D



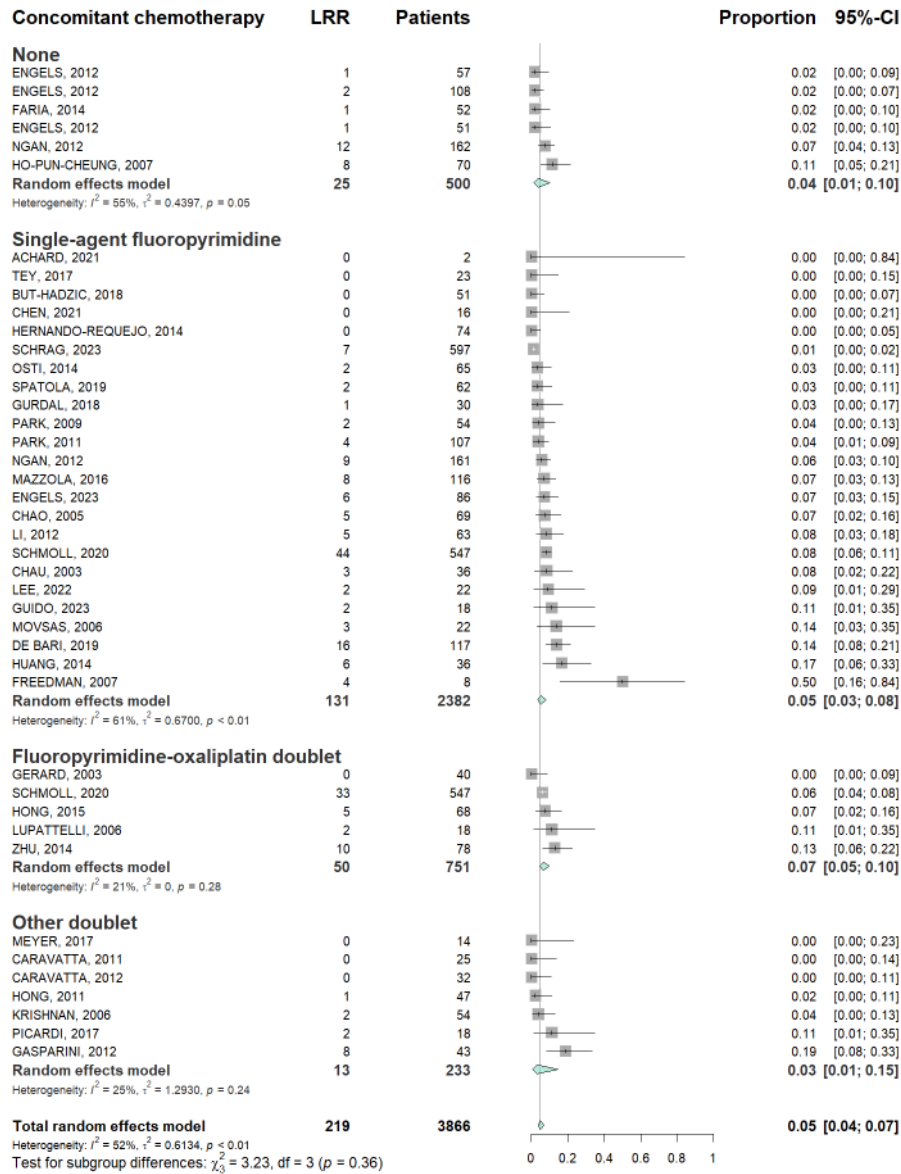
E



F



G



H

## Neo-adjuvant chemotherapy

LRR

Patients

Proportion

95%-CI

## None

|                             |            |             |                                                                                      |                          |
|-----------------------------|------------|-------------|--------------------------------------------------------------------------------------|--------------------------|
| ACHARD, 2021                | 0          | 2           |    | 0.00 [0.00; 0.84]        |
| TEY, 2017                   | 0          | 23          |     | 0.00 [0.00; 0.15]        |
| BUT-HADZIC, 2018            | 0          | 51          |     | 0.00 [0.00; 0.07]        |
| CARAVATTA, 2011             | 0          | 25          |     | 0.00 [0.00; 0.14]        |
| CARAVATTA, 2012             | 0          | 46          |     | 0.00 [0.00; 0.08]        |
| GERARD, 2003                | 0          | 40          |     | 0.00 [0.00; 0.09]        |
| HERNANDO-REQUEJO, 2014      | 0          | 74          |     | 0.00 [0.00; 0.05]        |
| SCHRAG, 2023                | 7          | 597         |     | 0.01 [0.00; 0.02]        |
| ENGELS, 2012                | 2          | 108         |     | 0.02 [0.00; 0.07]        |
| FARIA, 2014                 | 1          | 52          |     | 0.02 [0.00; 0.10]        |
| GENG, 2021                  | 1          | 52          |     | 0.02 [0.00; 0.10]        |
| HONG, 2011                  | 1          | 47          |     | 0.02 [0.00; 0.11]        |
| OSTI, 2014                  | 2          | 65          |     | 0.03 [0.00; 0.11]        |
| GURDAL, 2018                | 1          | 30          |     | 0.03 [0.00; 0.17]        |
| PARK, 2009                  | 2          | 54          |     | 0.04 [0.00; 0.13]        |
| KRISHNAN, 2006              | 2          | 54          |     | 0.04 [0.00; 0.13]        |
| PARK, 2011                  | 4          | 107         |     | 0.04 [0.01; 0.09]        |
| ZHAO, 2019                  | 7          | 141         |     | 0.05 [0.02; 0.10]        |
| ENGELS, 2023                | 9          | 168         |     | 0.05 [0.02; 0.10]        |
| KIM, 2016                   | 10         | 165         |     | 0.06 [0.03; 0.11]        |
| NGAN, 2012                  | 21         | 323         |     | 0.07 [0.04; 0.10]        |
| MAZZOLA, 2016               | 8          | 116         |    | 0.07 [0.03; 0.13]        |
| SCHMOLL, 2020               | 77         | 1094        |   | 0.07 [0.06; 0.09]        |
| CHAO, 2005                  | 5          | 69          |   | 0.07 [0.02; 0.16]        |
| HONG, 2015                  | 5          | 68          |   | 0.07 [0.02; 0.16]        |
| LI, 2012                    | 5          | 63          |   | 0.08 [0.03; 0.18]        |
| LEE, 2022                   | 2          | 22          |   | 0.09 [0.01; 0.29]        |
| PICARDI, 2017               | 2          | 18          |   | 0.11 [0.01; 0.35]        |
| GUIDO, 2023                 | 2          | 18          |   | 0.11 [0.01; 0.35]        |
| LUPATTELLI, 2006            | 2          | 18          |   | 0.11 [0.01; 0.35]        |
| HO-PUN-CHEUNG, 2007         | 8          | 70          |   | 0.11 [0.05; 0.21]        |
| MOVSAS, 2006                | 3          | 22          |   | 0.14 [0.03; 0.35]        |
| DE BARI, 2019               | 16         | 117         |   | 0.14 [0.08; 0.21]        |
| HUANG, 2014                 | 6          | 36          |   | 0.17 [0.08; 0.33]        |
| FREEDMAN, 2007              | 4          | 8           |  | 0.50 [0.16; 0.84]        |
| <b>Random effects model</b> | <b>215</b> | <b>3963</b> |   | <b>0.05 [0.03; 0.07]</b> |

Heterogeneity:  $I^2 = 52\%$ ,  $\tau^2 = 0.5172$ ,  $p < 0.01$ 

## Induction

|                             |           |            |                                                                                     |                          |
|-----------------------------|-----------|------------|-------------------------------------------------------------------------------------|--------------------------|
| SPATOLA, 2019               | 2         | 62         |  | 0.03 [0.00; 0.11]        |
| CHAU, 2003                  | 3         | 36         |  | 0.08 [0.02; 0.22]        |
| GASPARINI, 2012             | 8         | 43         |  | 0.19 [0.08; 0.33]        |
| <b>Random effects model</b> | <b>13</b> | <b>141</b> |  | <b>0.08 [0.03; 0.21]</b> |

Heterogeneity:  $I^2 = 67\%$ ,  $\tau^2 = 0.5172$ ,  $p = 0.05$ 

## Consolidation

|                             |           |           |                                                                                     |                          |
|-----------------------------|-----------|-----------|-------------------------------------------------------------------------------------|--------------------------|
| MEYER, 2017                 | 0         | 14        |  | 0.00 [0.00; 0.23]        |
| ZHU, 2014                   | 10        | 78        |  | 0.13 [0.06; 0.22]        |
| <b>Random effects model</b> | <b>10</b> | <b>92</b> |  | <b>0.08 [0.02; 0.26]</b> |

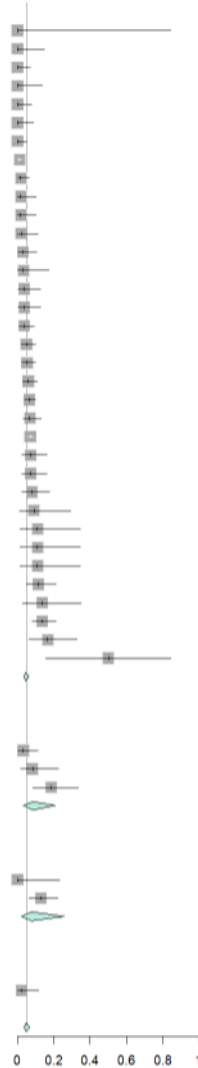
Heterogeneity:  $I^2 = 0\%$ ,  $\tau^2 = 0.5172$ ,  $p = 1.00$ 

## Induction and consolidation

|                |   |    |                                                                                     |                   |
|----------------|---|----|-------------------------------------------------------------------------------------|-------------------|
| LO GRECO, 2023 | 1 | 45 |  | 0.02 [0.00; 0.12] |
|----------------|---|----|-------------------------------------------------------------------------------------|-------------------|

## Total random effects model

|            |             |                                                                                     |                          |
|------------|-------------|-------------------------------------------------------------------------------------|--------------------------|
| <b>239</b> | <b>4241</b> |  | <b>0.05 [0.04; 0.07]</b> |
|------------|-------------|-------------------------------------------------------------------------------------|--------------------------|

Heterogeneity:  $I^2 = 53\%$ ,  $\tau^2 = 0.6029$ ,  $p < 0.01$ Residual heterogeneity:  $I^2 = 52\%$ ,  $\tau^2 = 0.5172$ ,  $p < 0.01$ Test for subgroup differences:  $\chi^2_3 = 0.77$ ,  $df = 3$  ( $p = 0.52$ )

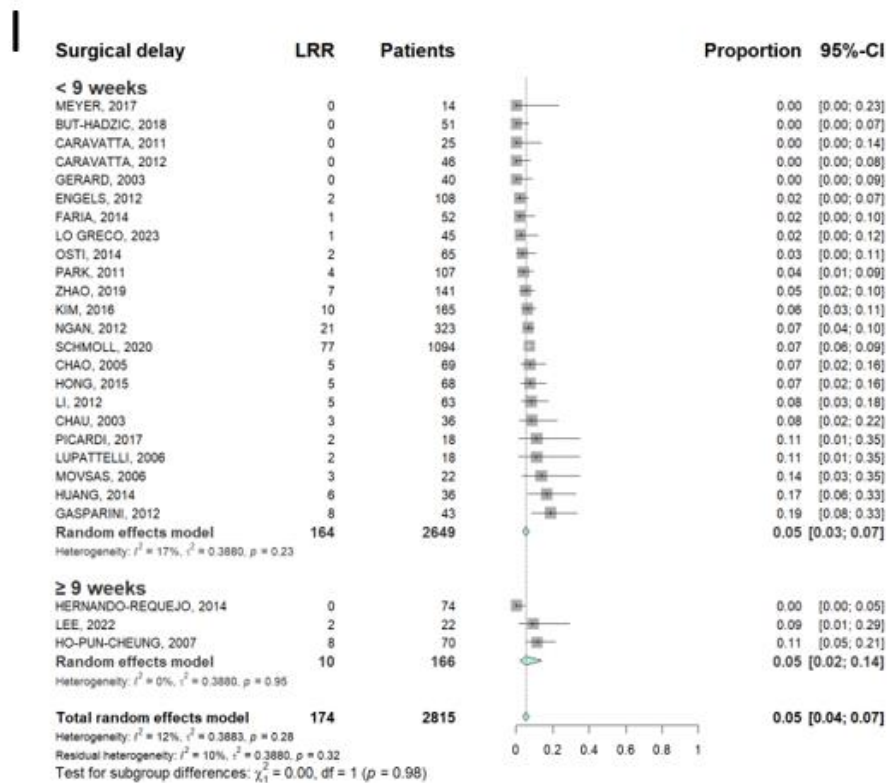
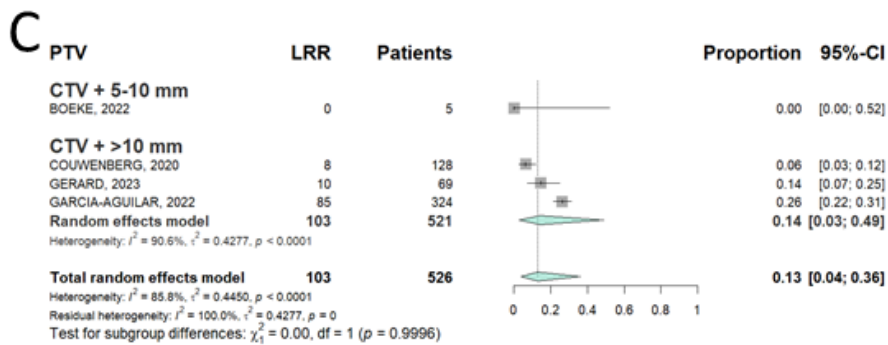
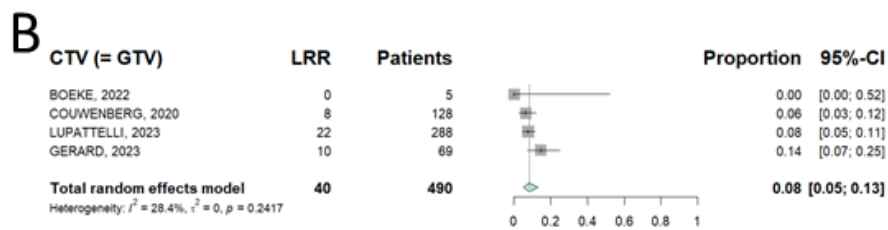
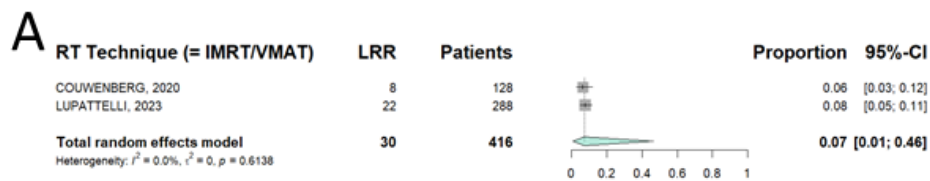
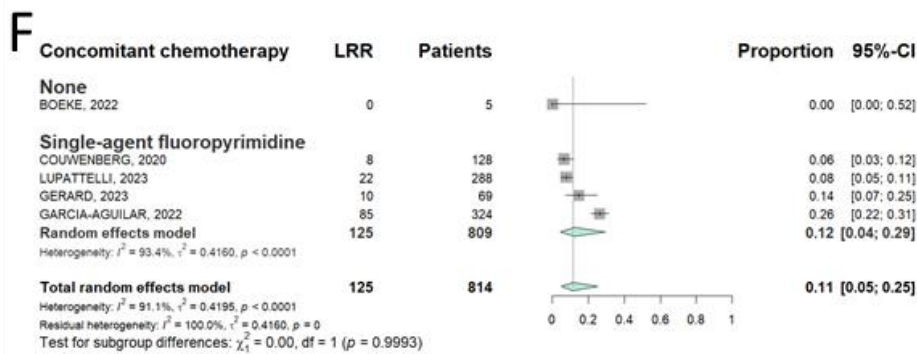
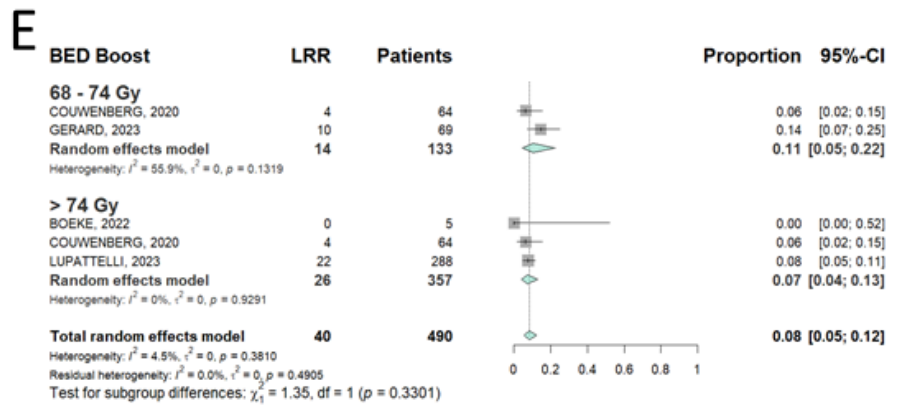
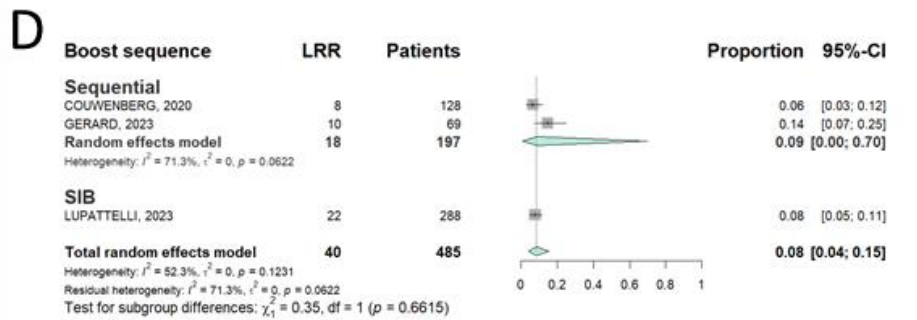


Figure A7.8: Details of the meta-analysis of studies with planned surgery reporting local recurrence rate as function of (A) Publication year, (B) RT modulation, (C) CTV definition, (d) PTV definition, (E) boost sequence, (F) boost BED, (G) concomitant chemotherapy, (H) neo-adjuvant chemotherapy, and (I) Surgical delay. The “LRR” column is the number of local recurrence rate events within each study, the “Patients” column reported the total of the patients within each study among the pooled publications, the “Proportion” column is the local recurrence rate within each study, the “95%-CI” column is the 95% confidence interval of the local recurrence rate within each study. 3D: Three-dimensional radiotherapy, BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, LRR: Local recurrence rate, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

### 7.7.2.4.3. Local recurrence rate results in «Watch and Wait» studies







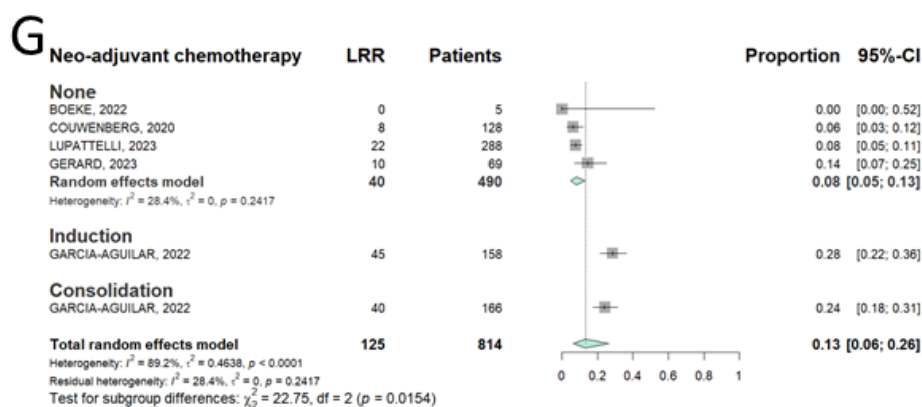


Figure A7.9: Details of the meta-analysis of «Watch and Wait» studies reporting local recurrence rate as function of (A) RT modulation, (B) CTV definition, (C) PTV definition, (D) boost sequence, (E) boost BED, (F) concomitant chemotherapy, and (G) neo-adjuvant chemotherapy. The “LRR” column is the number of local recurrence rate events within each study, the “Patients” column reported the total of the patients within each study among the pooled publications, the “Proportion” column is the local recurrence rate rate within each study, the “95%-CI” column is the 95% confidence interval of the local recurrence rate rate within each study. BED: Biologically effective dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, LRR: Local recurrence rate, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

### 7.7.3. Meta regression model of pathologic complete response as a function of radiotherapy (RT) dose.

#### 7.7.3.1. Meta regression methods

For cohorts reporting outcomes for a unique RT boost dose, a meta-regression model with the RT boost dose as a continuous predictor of the

pathological complete response was performed. In case of significant model, a non-linear correlation model would be used to determine pathological complete response probability as function of the RT boost dose using a sigmoidal function.

### 7.7.3.2. *Meta regression results*

The meta-regression model highlights that the boost biologically effective dose (BED) was significantly associated with a pathological complete response increase. The regression coefficient was 0.043 (95% confidence interval: 0.025 – 0.062, p-value < 0.001). The boost biologically effective dose explained 37.7% of the interstudy variance ( $R^2_*$  coefficient). After adjusting for boost biologically effective dose, moderate heterogeneity remained ( $I^2 = 65.6\%$ ). Using a non-linear correlation model, the pathological complete response rate could be approximated using the following formula (Figure A7.10):

$$pCR\ rate = \frac{1}{1 + e^{\frac{107.0 - Boost\ BED}{22.8}}} \quad Eq. A7.3$$

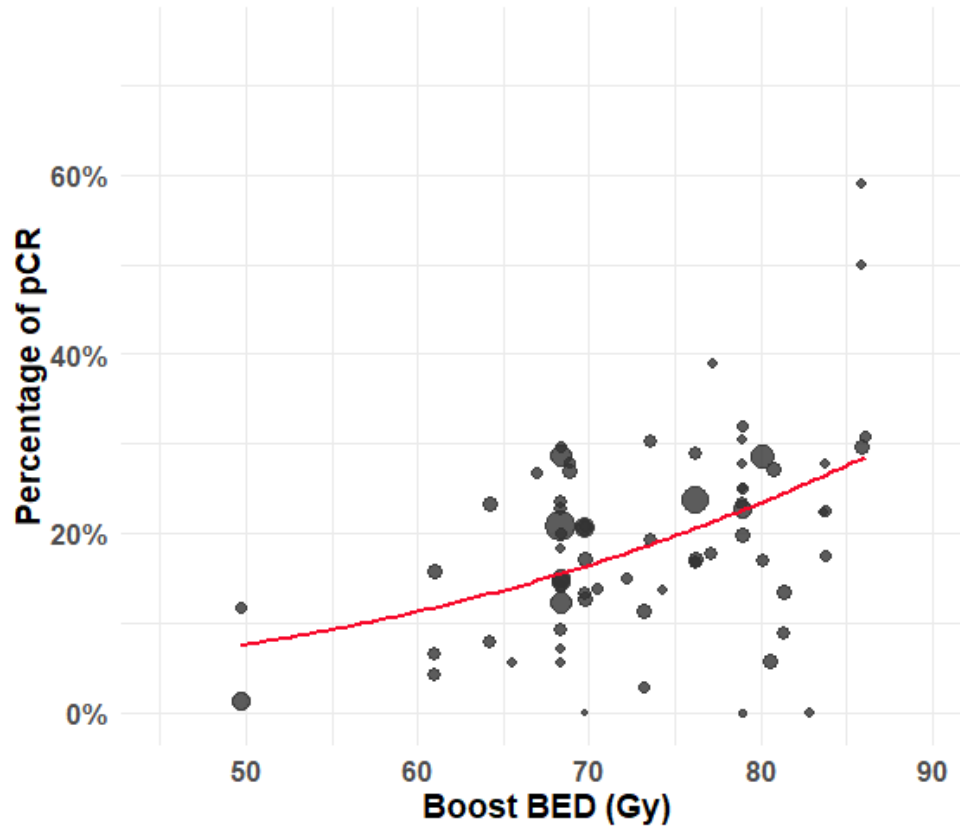


Figure A7.10: Reported pathological complete response rate as function of the boost biologically effective dose delivered to the primary tumour. Each dot represents one study and its size is function of the total population of this study. The red line reveals a non-linear positive correlation between pathological complete response and boost biologically effective dose. BED: Biologically effective dose, pCR: Pathologic complete response.

7.7.4. Supplementary Figures and Table

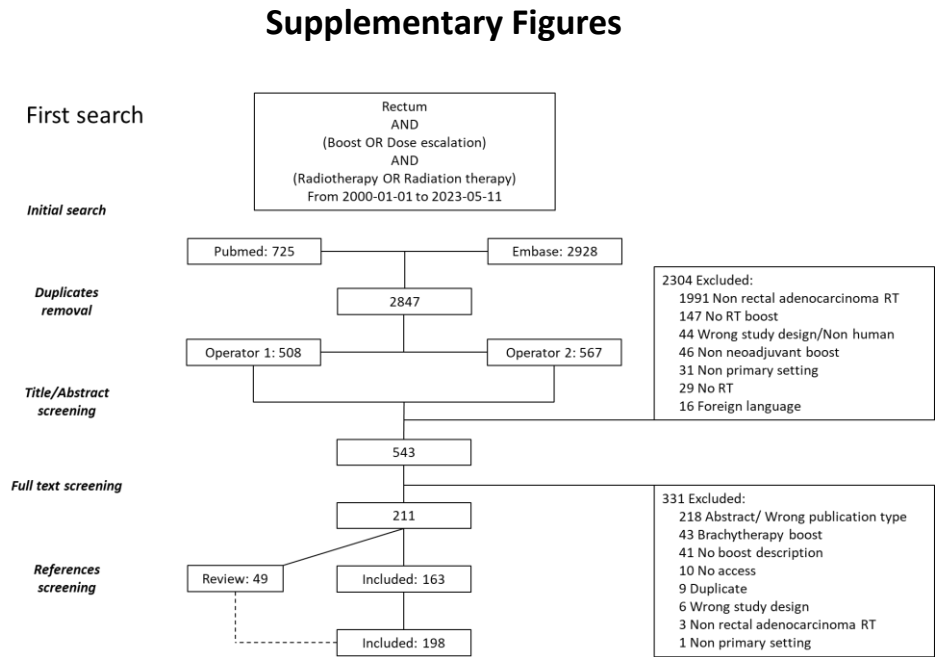


Figure A7.11: Flow chart of study selection at the first search. RT: Radiotherapy.

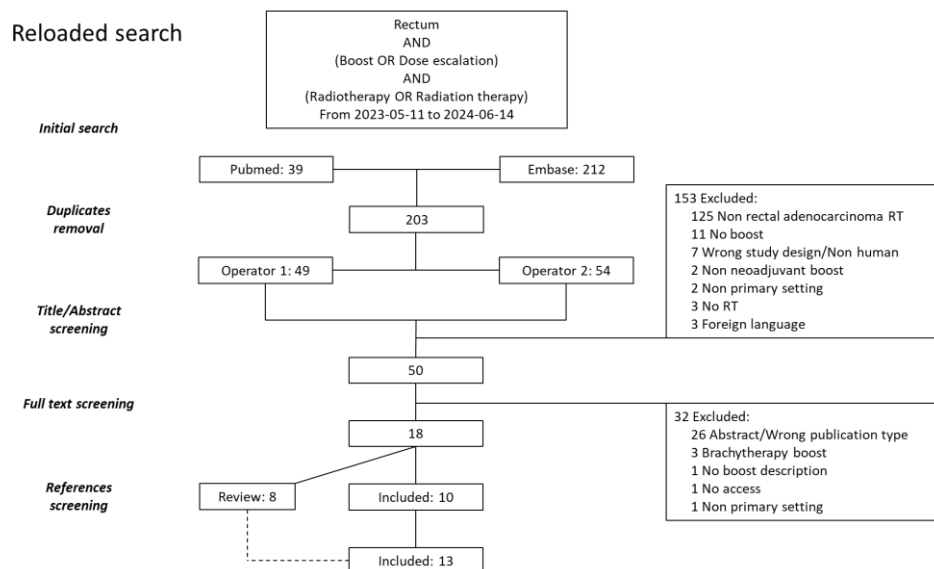


Figure A7.12: Flow chart of study selection after the search was reloaded. RT: Radiotherapy.

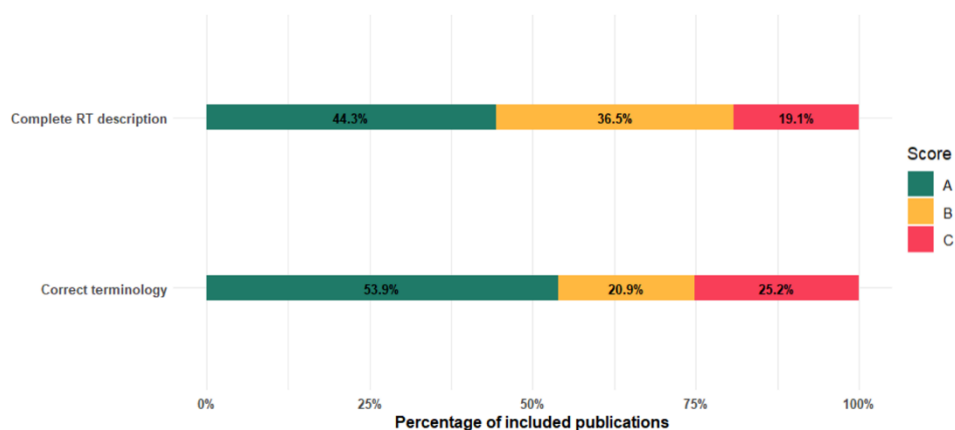


Figure A7.13: Results of the quality assessment analysis for the 115 studies included in the descriptive analysis after institutional duplicate removal. Studies that received a score of 'C' on at least one of these items were excluded, resulting in a total of 83 studies included in the descriptive analysis. RT: Radiotherapy.

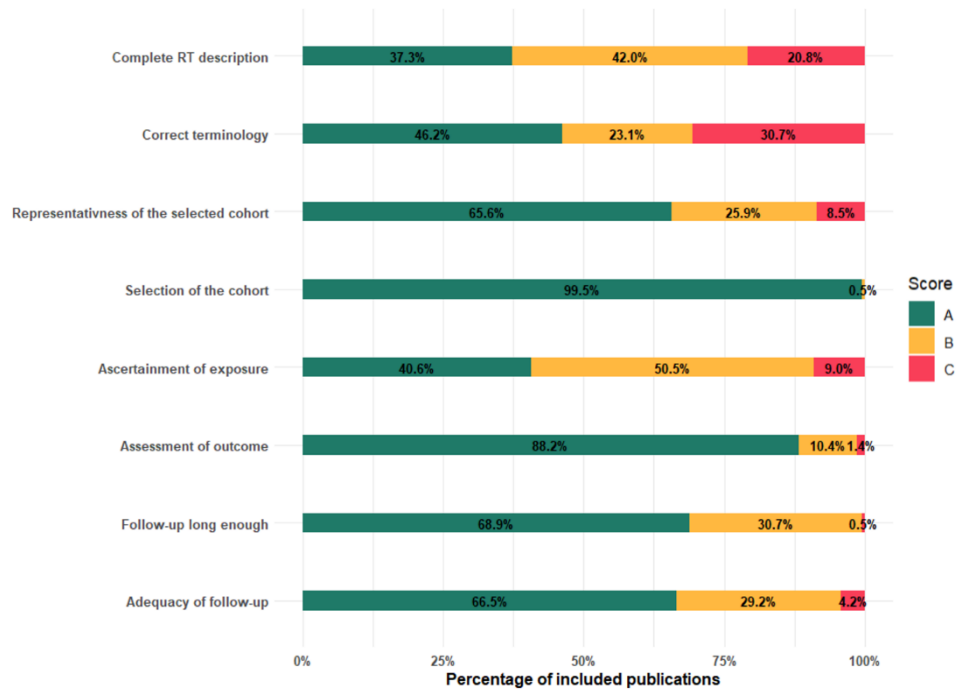
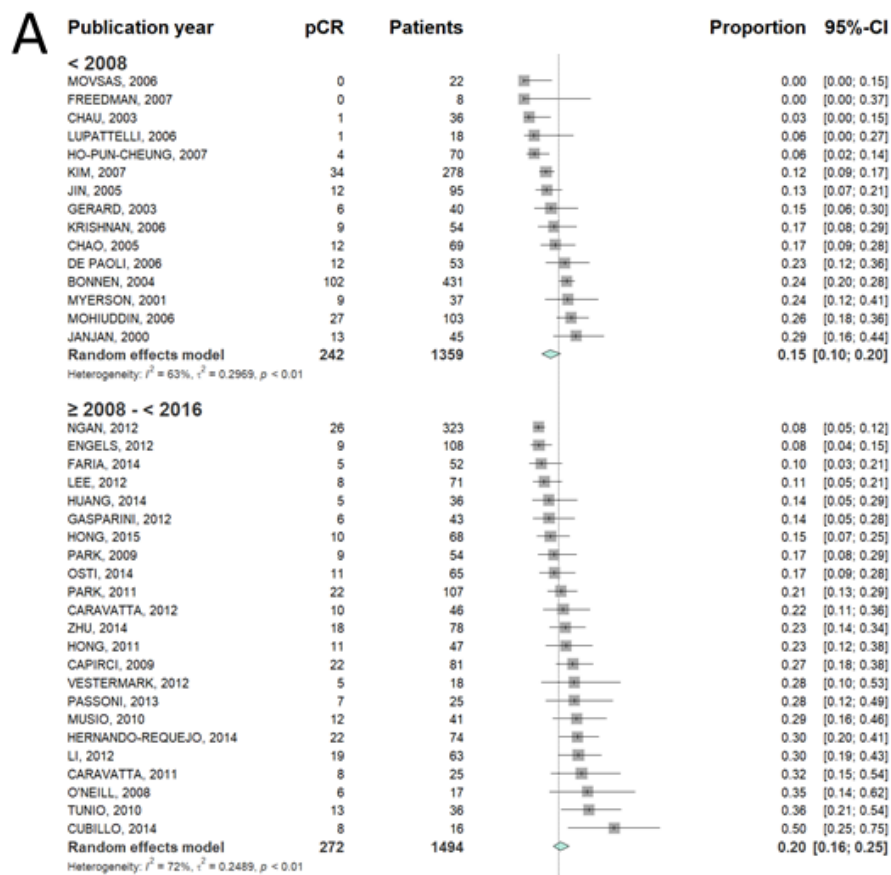
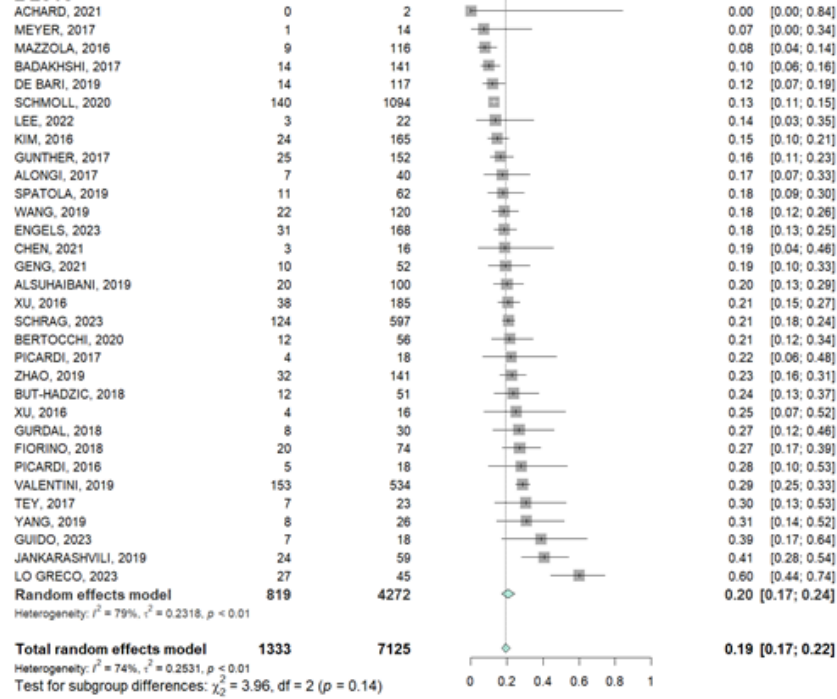


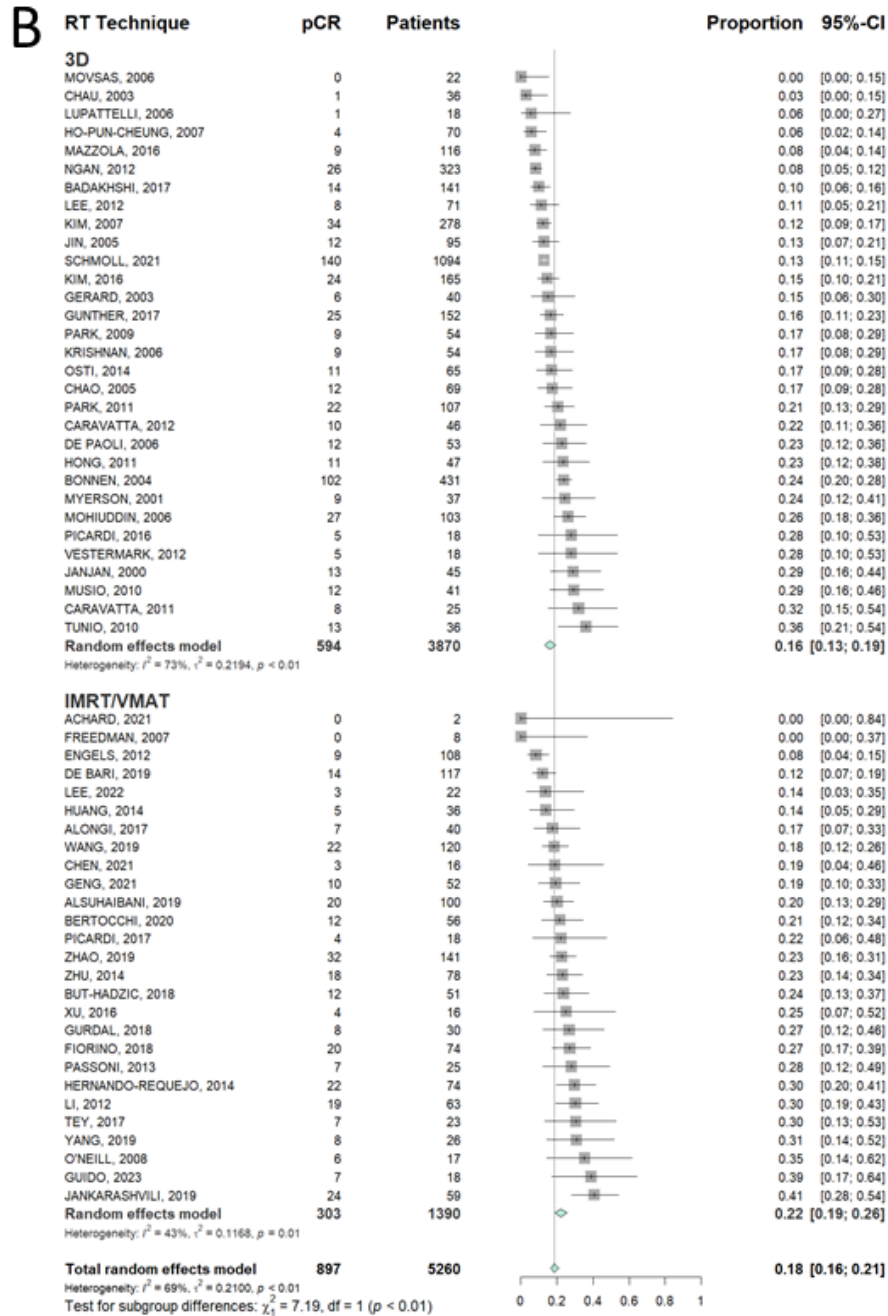
Figure A7.14: Results of the quality assessment analysis for the 141 studies with 212 different cohorts initially included in the meta-analysis. To ensure quality, studies with a 'C' score on any of the items were excluded, leaving 78 studies with 97 different cohorts for the meta-analysis. RT: Radiotherapy.

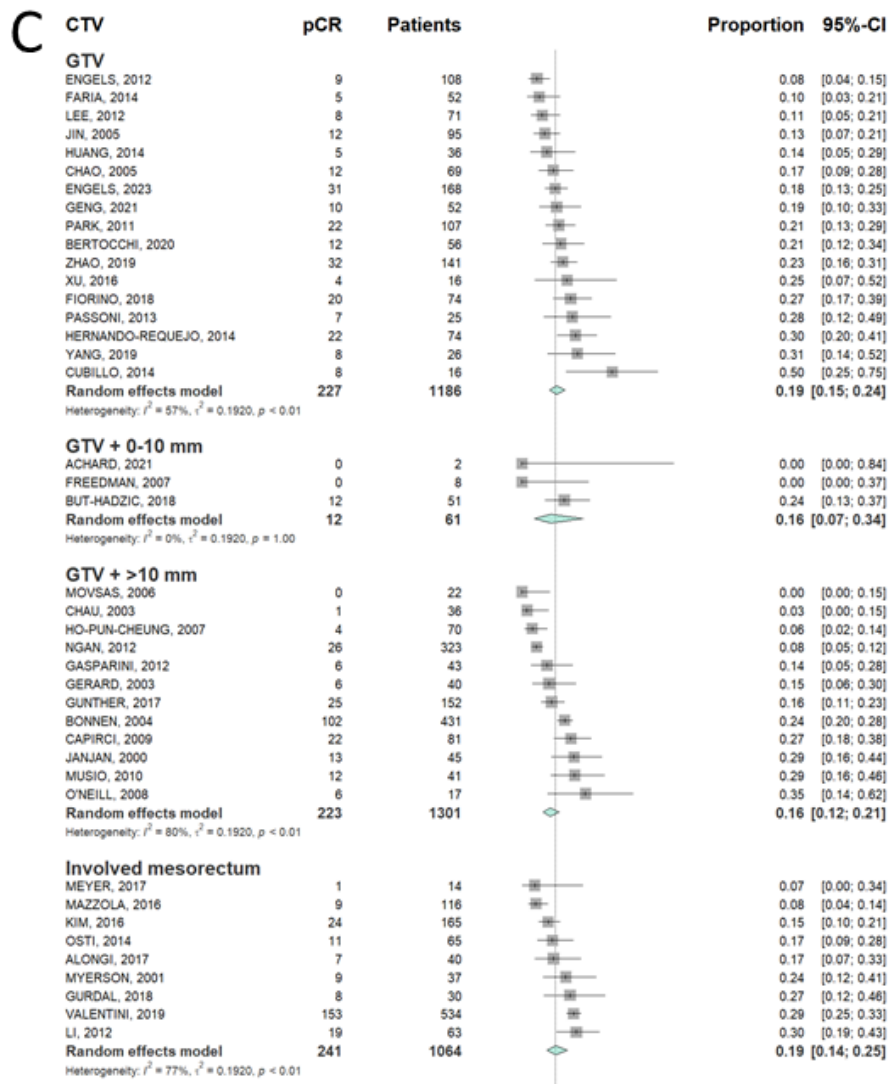


≥ 2016









# Larger than the involved mesorectum

|                             |            |             |
|-----------------------------|------------|-------------|
| LUPATTELLI, 2006            | 1          | 18          |
| DE BARI, 2019               | 14         | 117         |
| KIM, 2007                   | 34         | 278         |
| LEE, 2022                   | 3          | 22          |
| HONG, 2015                  | 10         | 68          |
| KRISHNAN, 2006              | 9          | 54          |
| ALSUHAIBANI, 2019           | 20         | 100         |
| CARAVATTA, 2012             | 10         | 46          |
| PICARDI, 2017               | 4          | 18          |
| DE PAOLI, 2006              | 12         | 53          |
| ZHU, 2014                   | 18         | 78          |
| HONG, 2011                  | 11         | 47          |
| MOHIUDDIN, 2006             | 27         | 103         |
| PICARDI, 2016               | 5          | 18          |
| TEY, 2017                   | 7          | 23          |
| CARAVATTA, 2011             | 8          | 25          |
| GUIDO, 2023                 | 7          | 18          |
| <b>Random effects model</b> | <b>200</b> | <b>1086</b> |

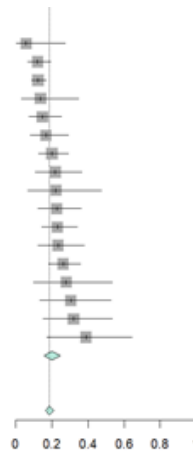
Heterogeneity:  $I^2 = 47\%$ ,  $\tau^2 = 0.1920$ ,  $p = 0.02$

**Total random effects model**      **903**      **4698**

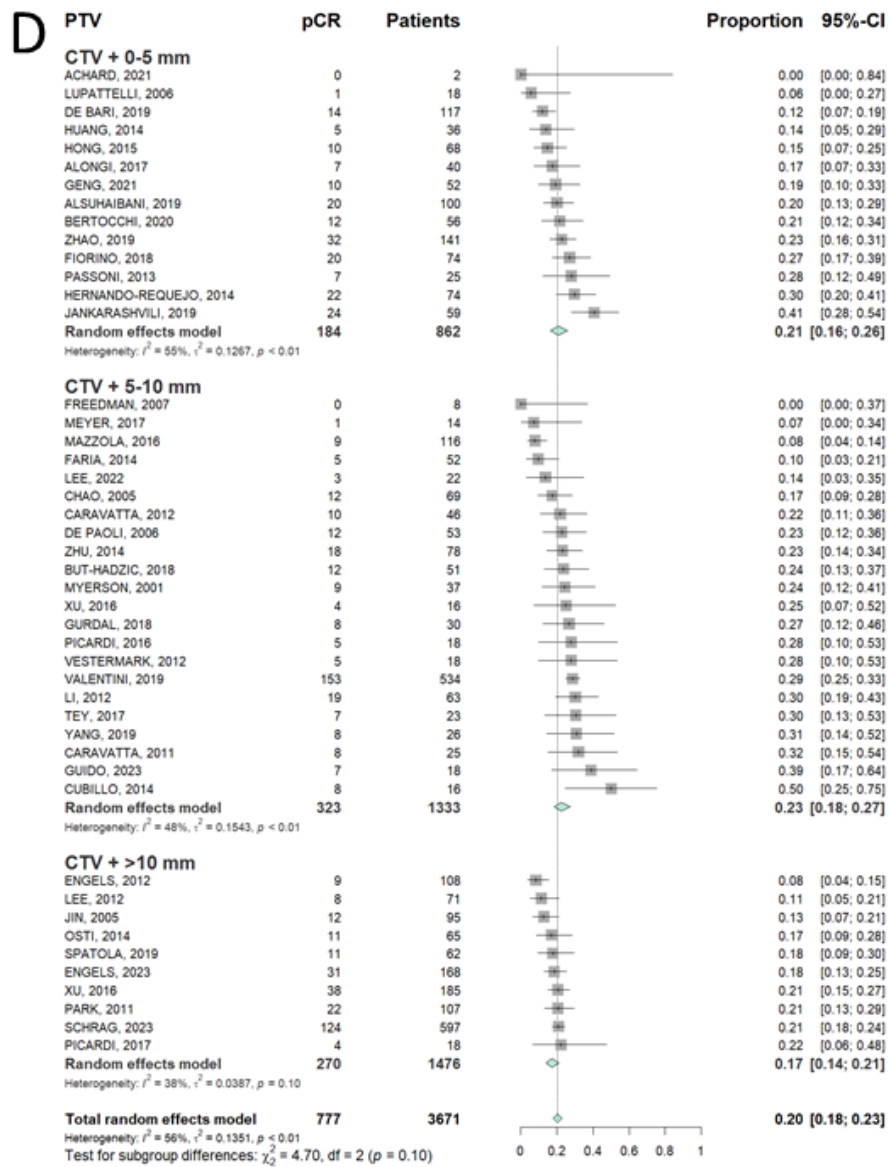
Heterogeneity:  $I^2 = 66\%$ ,  $\tau^2 = 0.1949$ ,  $p < 0.01$

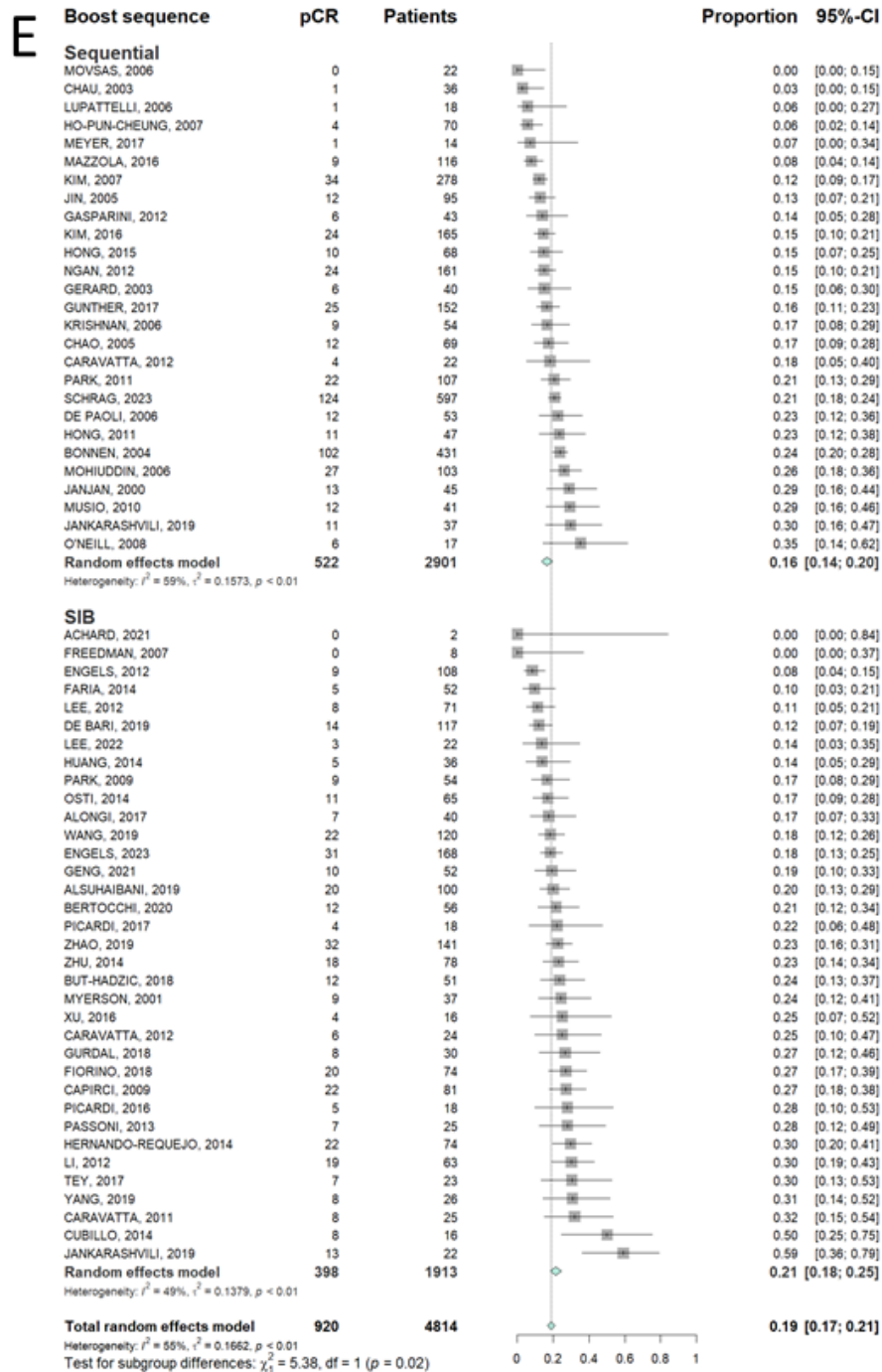
Residual heterogeneity:  $I^2 = 66\%$ ,  $\tau^2 = 0.1920$ ,  $p < 0.01$

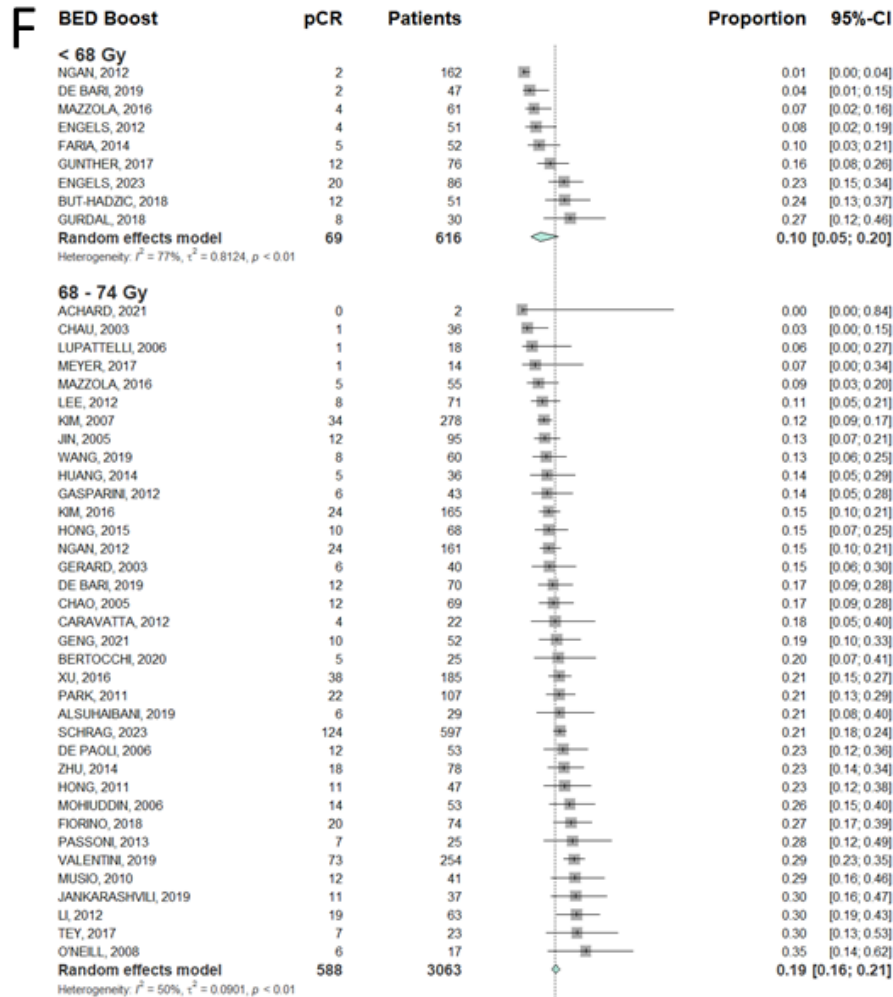
Test for subgroup differences:  $\chi^2_4 = 0.49$ ,  $df = 4$  ( $p = 0.75$ )



0.06 [0.00; 0.27]  
0.12 [0.07; 0.19]  
0.12 [0.09; 0.17]  
0.14 [0.03; 0.35]  
0.15 [0.07; 0.25]  
0.17 [0.08; 0.29]  
0.20 [0.13; 0.29]  
0.22 [0.11; 0.36]  
0.22 [0.06; 0.48]  
0.23 [0.12; 0.36]  
0.23 [0.14; 0.34]  
0.23 [0.12; 0.38]  
0.26 [0.18; 0.36]  
0.28 [0.10; 0.53]  
0.30 [0.13; 0.53]  
0.32 [0.15; 0.54]  
0.39 [0.17; 0.64]  
**0.20 [0.16; 0.25]**  
  
**0.19 [0.16; 0.21]**







# > 74 Gy

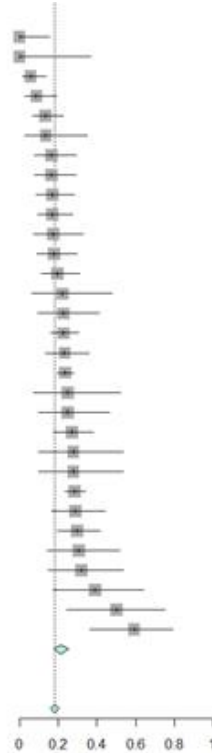
|                             |            |             |
|-----------------------------|------------|-------------|
| MOVSA, 2006                 | 0          | 22          |
| FREEDMAN, 2007              | 0          | 8           |
| HO-PUN-CHEUNG, 2007         | 4          | 70          |
| ENGELS, 2012                | 5          | 57          |
| ENGELS, 2023                | 11         | 82          |
| LEE, 2022                   | 3          | 22          |
| PARK, 2009                  | 9          | 54          |
| KRISHNAN, 2006              | 9          | 54          |
| OSTI, 2014                  | 11         | 65          |
| GUNTHER, 2017               | 13         | 76          |
| ALONGI, 2017                | 7          | 40          |
| SPATOLA, 2019               | 11         | 62          |
| ALSUHAIBANI, 2019           | 14         | 71          |
| PICARDI, 2017               | 4          | 18          |
| BERTOCCI, 2020              | 7          | 31          |
| ZHAO, 2019                  | 32         | 141         |
| WANG, 2019                  | 14         | 60          |
| BONNEN, 2004                | 102        | 431         |
| XU, 2016                    | 4          | 16          |
| CARAVATTA, 2012             | 6          | 24          |
| CAPRICI, 2009               | 22         | 81          |
| PICARDI, 2016               | 5          | 18          |
| VESTERMARK, 2012            | 5          | 18          |
| VALENTINI, 2019             | 80         | 280         |
| JANJAN, 2000                | 13         | 45          |
| HERNANDO-REQUEJO, 2014      | 22         | 74          |
| YANG, 2019                  | 8          | 26          |
| CARAVATTA, 2011             | 8          | 25          |
| GUIDO, 2023                 | 7          | 18          |
| CUBILLO, 2014               | 8          | 16          |
| JANKARASHVILI, 2019         | 13         | 22          |
| <b>Random effects model</b> | <b>457</b> | <b>2027</b> |

Heterogeneity:  $I^2 = 50\%$ ,  $\tau^2 = 0.2031$ ,  $p < 0.01$

**Total random effects model**      **1114**      **5706**

Heterogeneity:  $I^2 = 59\%$ ,  $\tau^2 = 0.2592$ ,  $p < 0.01$

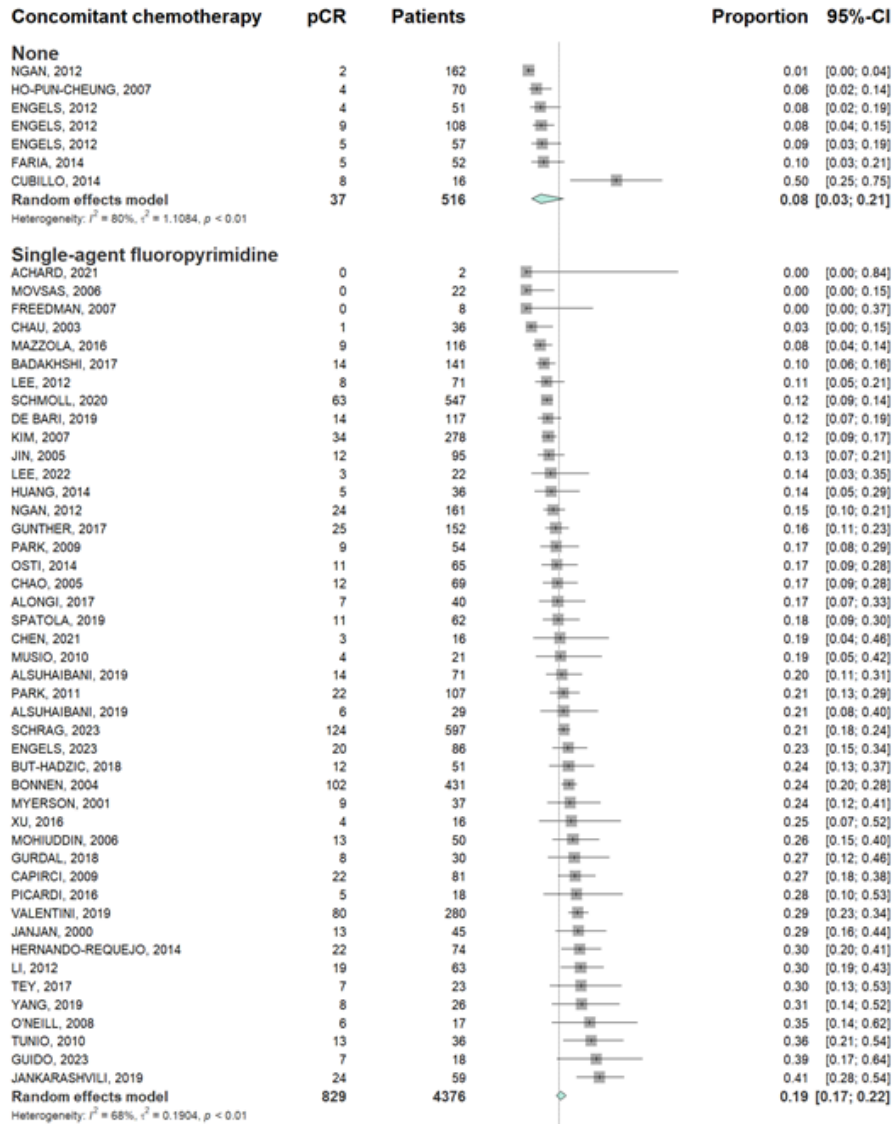
Test for subgroup differences:  $\chi^2_2 = 6.68$ ,  $df = 2$  ( $p = 0.04$ )



|             |                     |
|-------------|---------------------|
| 0.00        | [0.00; 0.15]        |
| 0.00        | [0.00; 0.37]        |
| 0.06        | [0.02; 0.14]        |
| 0.09        | [0.03; 0.19]        |
| 0.13        | [0.07; 0.23]        |
| 0.14        | [0.03; 0.35]        |
| 0.17        | [0.08; 0.29]        |
| 0.17        | [0.08; 0.29]        |
| 0.17        | [0.09; 0.28]        |
| 0.17        | [0.09; 0.27]        |
| 0.17        | [0.07; 0.33]        |
| 0.18        | [0.09; 0.30]        |
| 0.20        | [0.11; 0.31]        |
| 0.22        | [0.06; 0.48]        |
| 0.23        | [0.10; 0.41]        |
| 0.23        | [0.16; 0.31]        |
| 0.23        | [0.13; 0.36]        |
| 0.24        | [0.20; 0.28]        |
| 0.25        | [0.07; 0.52]        |
| 0.25        | [0.10; 0.47]        |
| 0.27        | [0.18; 0.38]        |
| 0.28        | [0.10; 0.53]        |
| 0.28        | [0.10; 0.53]        |
| 0.29        | [0.23; 0.34]        |
| 0.29        | [0.16; 0.44]        |
| 0.30        | [0.20; 0.41]        |
| 0.31        | [0.14; 0.52]        |
| 0.32        | [0.15; 0.54]        |
| 0.39        | [0.17; 0.64]        |
| 0.50        | [0.25; 0.75]        |
| 0.59        | [0.36; 0.79]        |
| <b>0.21</b> | <b>[0.18; 0.25]</b> |

**0.18 [0.16; 0.21]**

G





### Fluoropyrimidine-oxaliplatin doublet

|                             |            |             |             |                     |
|-----------------------------|------------|-------------|-------------|---------------------|
| LUPATTELLI, 2006            | 1          | 18          | 0.06        | [0.00; 0.27]        |
| SCHMOLL, 2020               | 77         | 547         | 0.14        | [0.11; 0.17]        |
| HONG, 2015                  | 10         | 68          | 0.15        | [0.07; 0.25]        |
| GERARD, 2003                | 6          | 40          | 0.15        | [0.06; 0.30]        |
| WANG, 2019                  | 22         | 120         | 0.18        | [0.12; 0.26]        |
| XU, 2016                    | 38         | 185         | 0.21        | [0.15; 0.27]        |
| ZHU, 2014                   | 18         | 78          | 0.23        | [0.14; 0.34]        |
| FIORINO, 2018               | 20         | 74          | 0.27        | [0.17; 0.39]        |
| VESTERMARK, 2012            | 5          | 18          | 0.28        | [0.10; 0.53]        |
| PASSONI, 2013               | 7          | 25          | 0.28        | [0.12; 0.49]        |
| VALENTINI, 2019             | 73         | 254         | 0.29        | [0.23; 0.35]        |
| MUSIO, 2010                 | 8          | 20          | 0.40        | [0.19; 0.64]        |
| <b>Random effects model</b> | <b>285</b> | <b>1447</b> | <b>0.21</b> | <b>[0.17; 0.26]</b> |

Heterogeneity:  $I^2 = 70\%$ ,  $\tau^2 = 0.0886$ ,  $p < 0.01$

### Other doublet

|                             |           |            |             |                     |
|-----------------------------|-----------|------------|-------------|---------------------|
| MEYER, 2017                 | 1         | 14         | 0.07        | [0.00; 0.34]        |
| GASPARINI, 2012             | 6         | 43         | 0.14        | [0.05; 0.28]        |
| KRISHNAN, 2006              | 9         | 54         | 0.17        | [0.08; 0.29]        |
| PICARDI, 2017               | 4         | 18         | 0.22        | [0.06; 0.48]        |
| HONG, 2011                  | 11        | 47         | 0.23        | [0.12; 0.38]        |
| CARAVATTA, 2012             | 8         | 32         | 0.25        | [0.11; 0.43]        |
| MOHIUDDIN, 2006             | 14        | 53         | 0.26        | [0.15; 0.40]        |
| CARAVATTA, 2011             | 8         | 25         | 0.32        | [0.15; 0.54]        |
| <b>Random effects model</b> | <b>61</b> | <b>286</b> | <b>0.21</b> | <b>[0.16; 0.28]</b> |

Heterogeneity:  $I^2 = 0\%$ ,  $\tau^2 = 0$ ,  $p = 0.51$

### Total random effects model

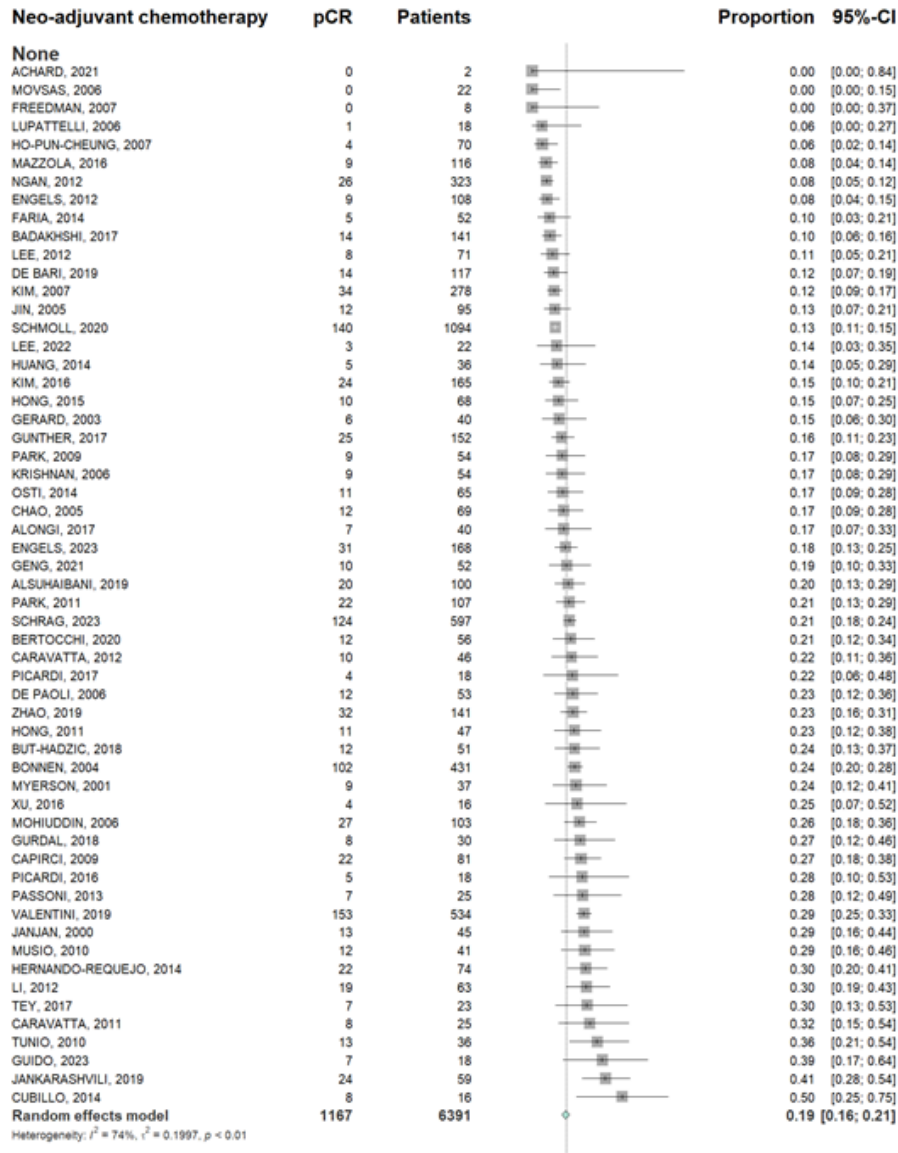
|  |             |             |             |                     |
|--|-------------|-------------|-------------|---------------------|
|  | <b>1212</b> | <b>6625</b> | <b>0.18</b> | <b>[0.16; 0.21]</b> |
|--|-------------|-------------|-------------|---------------------|

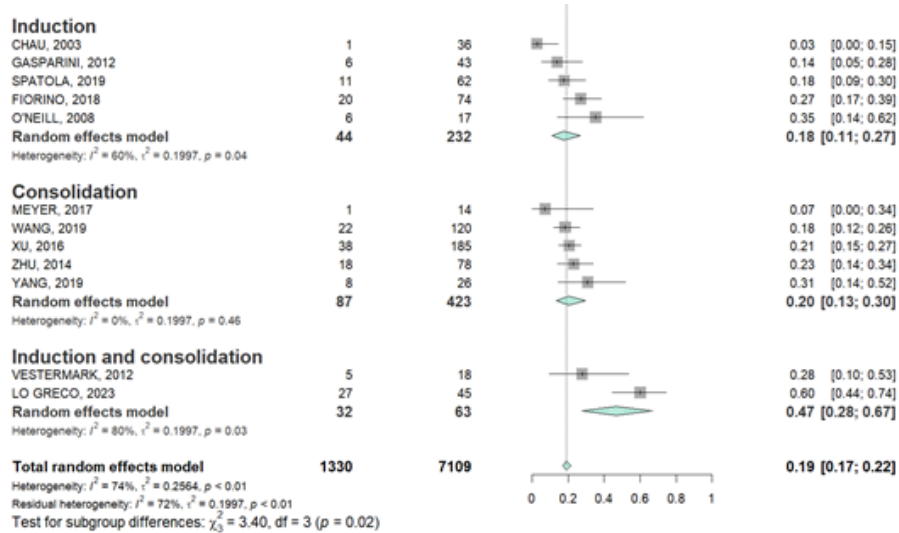
Heterogeneity:  $I^2 = 70\%$ ,  $\tau^2 = 0.2883$ ,  $p < 0.01$

Test for subgroup differences:  $\chi^2_3 = 6.26$ ,  $df = 3$  ( $p = 0.10$ )



# H





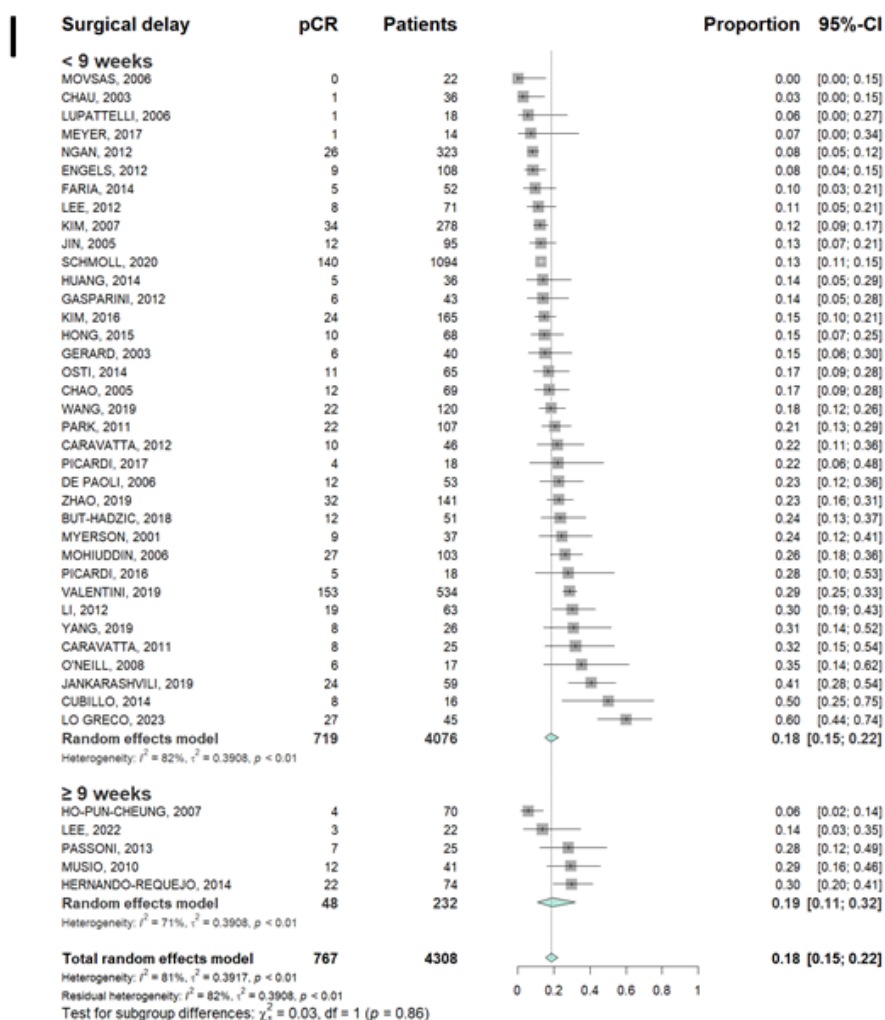


Figure A7.15: Details of the meta-analysis of studies reporting pathological complete response rate as function of (A) Publication year, (B) RT modulation, (C) CTV definition, (d) PTV definition, (E) boost sequence, (F) boost BED, (G) concomitant chemotherapy, (H) neo-adjuvant chemotherapy, and (I) Surgical delay. The “pCR” column is the number of pathological complete response events within each study, the “Patients” column reported the total of the patients within each study among the pooled publications, the “Proportion” column is the pathological complete response rate within each study, the “95%-CI” column is the 95% confidence interval of the

pathological complete response rate within each study. 3D: Three-dimensional radiotherapy, BED: Biological equivalent dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, pCR: Pathologic complete response, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.

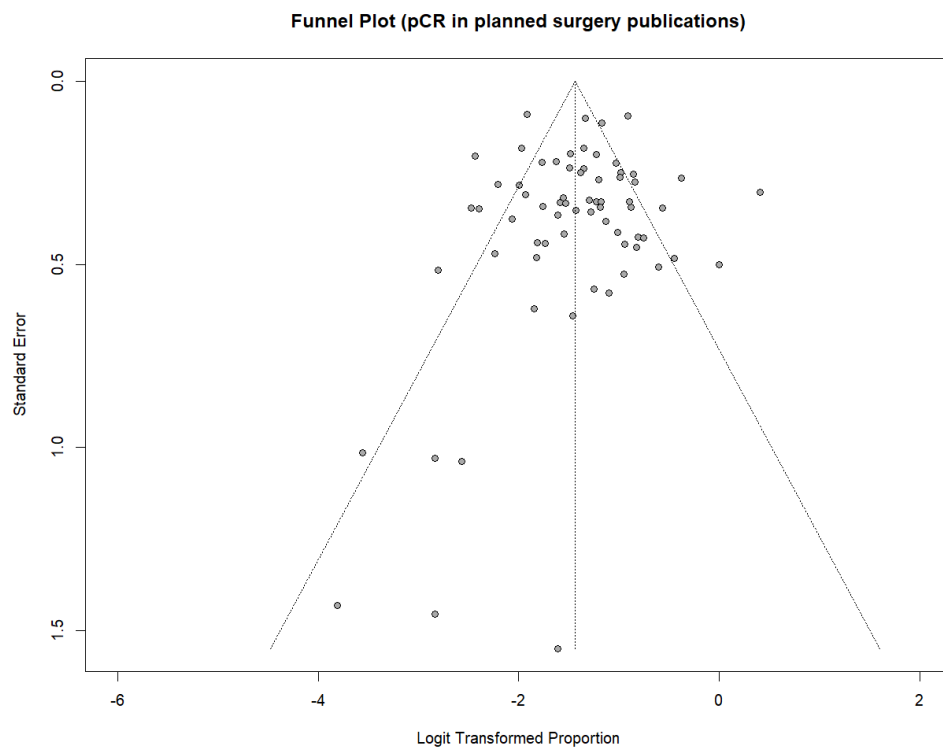


Figure A7.16: Funnel plot of planned surgery publications reporting pathological complete response. pCR: Pathologic complete response.

| Class of data  | Extracted characteristics  | Categories  |                        |                                           |                |                      |                |
|----------------|----------------------------|-------------|------------------------|-------------------------------------------|----------------|----------------------|----------------|
| Publication    | Author                     |             |                        |                                           |                |                      |                |
|                | Year                       | < 2008      | ≥ 2008 - < 2016        | ≥ 2016                                    |                |                      |                |
|                | Institution                |             |                        |                                           |                |                      |                |
|                | Study design               | In silico   | Protocol               | Retrospective                             | Prospective    |                      |                |
| RT technique   | Modulation                 | 3D          | IMRT/VMA<br>T          | 3D/IMRT/VM<br>AT                          | MRGRT          | 3D/IMRT/PT           |                |
|                | Boost timing               | Sequential  | Simultaneous           | Combination of sequential or simultaneous |                |                      |                |
|                | Bifractionated             | No          | Only for the boost     | Whole treatment                           |                |                      |                |
|                | Adaptive RT for boost      | No          | Yes                    |                                           |                |                      |                |
|                | Description of preparation | No          | Yes                    |                                           |                |                      |                |
| RT preparation | Oral preparation           | No contrast | Water as oral contrast | Oral contrast                             |                |                      |                |
|                | Rectum preparation         | No protocol | Empty rectum           | Filling protocol                          | Rectal Gel     | Rectal opacification |                |
|                | Bladder preparation        | None        | Comfortable full       | Full                                      | Water protocol | Baryum protocol      | Other protocol |
|                |                            |             |                        |                                           |                |                      |                |

|                      |                                            |            |                            |                     |                                         |                        |              |
|----------------------|--------------------------------------------|------------|----------------------------|---------------------|-----------------------------------------|------------------------|--------------|
| RT boost delineation | Intravenous contrast                       | No         | Yes                        |                     |                                         |                        |              |
|                      | Treatment position                         | Supine     | Prone                      | Both                |                                         |                        |              |
|                      | Description of boost volume                | No         | Yes                        |                     |                                         |                        |              |
|                      | Multi-modality imaging for GTV delineation | None       | Digital rectal examination | Endoscopy           | Ultrasonography                         | MRI                    | PET-CT       |
|                      | Description of CTV                         | No         | Yes                        |                     |                                         |                        |              |
|                      | CTV margin                                 | CTV is GTV | Numeric margin             | Anatomic margin     | Numeric and anatomic margin             |                        |              |
|                      | Numeric CTV margin value                   | 0 mm       | > 0 – 10 mm                | > 10 – 20 mm        | Anisotropic                             |                        |              |
|                      | Anatomic CTV margin                        | None       | Rectum involved            | Mesorectum involved | Mesorectum and presacral space involved | Entire presacral space | Other        |
|                      | Description of PTV                         | No         | None                       |                     |                                         |                        |              |
|                      | PTV is isotropic                           | No         | Yes                        |                     |                                         |                        |              |
|                      | PTV margin value*                          | 0 mm       | > 0 – 5 mm                 | > 5 – 10 mm         | > 10 – 15 mm                            | > 15 – 20 mm           | > 20 – 30 mm |

|              |                                  |                            |                  |                             |                                            |                                                     |                                                 |       |  |
|--------------|----------------------------------|----------------------------|------------------|-----------------------------|--------------------------------------------|-----------------------------------------------------|-------------------------------------------------|-------|--|
| RT dose      | <i>N fractions</i>               | < 10                       | 10 – 20          | > 20                        |                                            |                                                     |                                                 |       |  |
|              | <i>BED elective</i>              | < 68 Gy                    | 68 – 74 Gy       | > 74 Gy                     |                                            |                                                     |                                                 |       |  |
|              | <i>BED boost</i>                 | < 68 Gy                    | 68 – 74 Gy       | > 74 Gy                     |                                            |                                                     |                                                 |       |  |
| Chemotherapy | <i>Concomitant chemotherapy</i>  | None                       | 5-fluorouracile  | Capecitabine                | 5-fluorouracile/Capecitabine + oxaliplatin | 5-fluorouracile/Capecitabine + another chemotherapy | 5-fluorouracile/Capecitabine + targeted therapy | Other |  |
|              | <i>Neo-adjuvant chemotherapy</i> | Induction                  | Consolidation    | Induction and consolidation |                                            |                                                     |                                                 |       |  |
| Follow-up    | <i>Follow-up strategy</i>        | Scheduled surgery          | Watch and wait   |                             |                                            |                                                     |                                                 |       |  |
|              | <i>Watch and wait management</i> | Digital rectal examination | Endoscopy        | MRI                         | PET-CT                                     | CT                                                  | Ultrasonography                                 |       |  |
|              | <i>Surgical delay</i>            | < 9 weeks                  | ≥ 9 weeks        |                             |                                            |                                                     |                                                 |       |  |
|              | <i>Follow-up duration</i>        | < 12 months                | > 12 – 24 months | > 24 – 36 months            | > 36 – 60 months                           | > 60 months                                         |                                                 |       |  |

Table A7.5: Rectal boost and treatment parameters extracted. \* In case of anisotropic PTV margin, the highest value is reported.



| Score    | Complete RT description                                                                                                                                                              | Correct terminology | Representativeness of the selected cohort                | Selection of the cohort                     | Ascertainment of exposure                    | Assessment of outcome                    | Follow-up long enough                                     | Adequacy of follow up                                            |
|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|----------------------------------------------------------|---------------------------------------------|----------------------------------------------|------------------------------------------|-----------------------------------------------------------|------------------------------------------------------------------|
| <b>C</b> | Only dose is reported                                                                                                                                                                | Totally inaccurate  | No locally advanced rectal cancer                        | No description                              | Not reported or >15% of patients not treated | No description                           | <1 year or not reported if local recurrence rate reported | No description of surgical delay and long-term follow-up         |
| <b>B</b> | Incomplete RT description                                                                                                                                                            | Some inaccuracy     | Combination of locally advanced and other rectal cancers | Cohorts from other institutions or database | Not all patients treated (up to 15%)         | Different assessments between patients   | 1-3 years if local recurrence rate reported               | Incomplete description of surgical delay and long-term follow-up |
| <b>A</b> | Complete RT description (Including preparation, target volume description, multi-modality imaging use, RT modulation, prescribed dose, concomitant chemotherapy, and surgical delay) | Accurate            | Only locally advanced rectal cancer                      | Retrospective or prospective                | All patients treated                         | One identical assesment for all patients | >3 years if local recurrence rate reported                | Precise description of surgical delay and long-term follow-up    |

*Table A7.6: Modified Newcastle - Ottawa quality assessment scale for cohort studies.*

| Author, year            | Study design  | RT modulation | Boost sequence | Position | CTV                         | PTV is Isotropic | Concomitant chemotherapy | Strategy         | Surgical delay/ Evaluation of the cCR in «Watch and Wait» |
|-------------------------|---------------|---------------|----------------|----------|-----------------------------|------------------|--------------------------|------------------|-----------------------------------------------------------|
| Achard, 2021 [335]      | Retrospective | IMRT/VMAT     | SIB            | Supine   | Numeric margin              | Yes              | Capecitabine             | Planned surgery  | Out of categories                                         |
| Al-Khalidi, 2022 [336]  | In silico     | 3D            | Sequential     | Prone    | Numeric and anatomic margin | Yes              | -                        | -                | -                                                         |
| Alsuhailani, 2019 [337] | Retrospective | IMRT/VMAT     | SIB            | -        | Numeric and anatomic margin | Yes              | Capecitabine             | Planned surgery  | Out of categories                                         |
| Anderson, 2007 [338]    | In silico     | 3D            | Sequential     | Prone    | Numeric and anatomic margin | Yes              | 5-fluorouracil           | -                | -                                                         |
| Appelt, 2015 [339]      | Prospective   | IMRT/VMAT     | SIB            | Supine   | Anatomic margin             | Yes              | Other Singlet            | W&W              | 6 weeks                                                   |
| Arp, 2024 [340]         | In silico     | IMRT/VMAT     | SIB            | Supine   | Anatomic margin             | No               | -                        | «Watch and Wait» | 6 – 12 weeks                                              |
| Badakhshi, 2017 [341]   | Retrospective | 3D            | Sequential     | Prone    | -                           | -                | 5-fluorouracil           | Planned surgery  | Out of categories                                         |

|                           |               |                  |                      |                       |                                   |     |                |                        |                   |
|---------------------------|---------------|------------------|----------------------|-----------------------|-----------------------------------|-----|----------------|------------------------|-------------------|
| Bae, 2017 [342]           | Retrospective | 3D/IMRT/V<br>MAT | Sequential           | Prone                 | Numeric and<br>anatomic<br>margin | Yes | 5-fluorouracil | Planned<br>surgery     | Out of categories |
| Bakkal, 2021<br>[343]     | In<br>silico  | 3D/IMRT/V<br>MAT | Sequential<br>or SIB | Supine                | Numeric<br>margin                 | Yes | -              | -                      | -                 |
| Ballonoff, 2008<br>[344]  | Prospective   | IMRT/VMAT        | SIB                  | Prone                 | No margin                         | Yes | Capecitabine   | Planned<br>surgery     | < 9 weeks         |
| Bassi, 2008<br>[345]      | In<br>silico  | -                | -                    | Prone<br>or<br>supine | No margin                         | -   | -              | -                      | -                 |
| Bertocchi, 2020<br>[346]  | Prospective   | IMRT/VMAT        | SIB                  | Supine                | No margin                         | Yes | -              | Planned<br>surgery     | Out of categories |
| Boeke, 2022<br>[347]      | Prospective   | MRI-guided<br>RT | Sequential<br>or SIB | -                     | No margin                         | Yes | None           | «Watch<br>and<br>Wait» | 3 months          |
| But-Hadzic,<br>2018 [348] | Prospective   | IMRT/VMAT        | SIB                  | -                     | Numeric<br>margin                 | No  | Capecitabine   | Planned<br>surgery     | < 9 weeks         |
| Capirci, 2009<br>[349]    | Prospective   | -                | SIB                  | Prone                 | Numeric<br>margin                 | -   | 5-fluorouracil | Planned<br>surgery     | Out of categories |
| Chen, 2020<br>[350]       | Retrospective | 3D/IMRT/V<br>MAT | Sequential           | Prone                 | -                                 | No  | 5-fluorouracil | Planned<br>surgery     | < 9 weeks         |

|                         |               |                       |            |        |                             |     |                                    |                  |                   |
|-------------------------|---------------|-----------------------|------------|--------|-----------------------------|-----|------------------------------------|------------------|-------------------|
| Chen, 2021<br>[351]     | Retrospective | IMRT/VMAT             | SIB        | -      | -                           | -   | Capecitabine                       | Planned surgery  | -                 |
| Chiloiro, 2023<br>[334] | Prospective   | MRI-guided RT         | SIB        | -      | No margin                   | Yes | 5-fluorouracil                     | «Watch and Wait» | 6 – 8 weeks       |
| Colaco, 2014<br>[352]   | In silico     | 3D/IMRT/Protontherapy | Sequential | Prone  | No margin                   | Yes | -                                  | -                | -                 |
| De Bari, 2019<br>[353]  | Retrospective | IMRT/VMAT             | SIB        | -      | Numeric and anatomic margin | Yes | Capecitabine                       | Planned surgery  | Out of categories |
| De Paoli, 2006<br>[354] | Prospective   | 3D                    | Sequential | Prone  | Numeric and anatomic margin | Yes | Capecitabine                       | Planned surgery  | < 9 weeks         |
| Devlin, 2022<br>[355]   | In silico     | IMRT/VMAT             | SIB        | Supine | Numeric margin              | Yes | -                                  | -                | -                 |
| Engels, 2023<br>[356]   | Prospective   | -                     | SIB        | -      | No margin                   | No  | None                               | Planned surgery  | Out of categories |
| Faria, 2014<br>[357]    | Prospective   | 3D/IMRT/VMAT          | SIB        | -      | No margin                   | Yes | None                               | Planned surgery  | < 9 weeks         |
| Fiorino, 2018<br>[358]  | Prospective   | IMRT/VMAT             | SIB        | Supine | No margin                   | Yes | 5-fluorouracil-oxaliplatin doublet | «Watch and Wait» | -                 |

|                            |               |              |                   |                 |                             |     |                                    |                  |                   |
|----------------------------|---------------|--------------|-------------------|-----------------|-----------------------------|-----|------------------------------------|------------------|-------------------|
| Fleischmann, 2022 [359]    | Protocol      | IMRT/VMAT    | Sequential        | -               | Numeric margin              | -   | Other-doublet                      | «Watch and Wait» | 10 weeks          |
| Garant, 2022 [360]         | Prospective   | 3D           | Sequential        | -               | Numeric margin              | Yes | 5-fluorouracil                     | «Watch and Wait» | 13 weeks          |
| Garcia-Aguilar, 2022 [103] | Prospective   | 3D/IMRT/VMAT | Sequential or SIB | Prone or supine | Anatomic margin             | No  | 5-fluorouracil                     | «Watch and Wait» | 8 +/- 4 weeks     |
| Gasparini, 2012 [361]      | Prospective   | -            | Sequential        | -               | Numeric margin              | -   | Other-doublet                      | Planned surgery  | < 9 weeks         |
| Geng, 2021 [362]           | Retrospective | IMRT/VMAT    | SIB               | Supine          | No margin                   | Yes | Capecitabine                       | Planned surgery  | Out of categories |
| Gerard, 2003 [363]         | Prospective   | 3D           | Other             | Prone           | Numeric margin              | -   | 5-fluorouracil-oxaliplatin doublet | Planned surgery  | < 9 weeks         |
| Gerard, 2023 [121]         | Prospective   | 3D/IMRT/VMAT | Sequential        | Prone or supine | No margin                   | Yes | Capecitabine                       | «Watch and Wait» | 24 weeks          |
| Guido, 2023 [364]          | Prospective   | IMRT/VMAT    | Sequential or SIB | Supine          | Numeric and anatomic margin | Yes | Capecitabine                       | Planned surgery  | Out of categories |

|                                 |               |               |            |                 |                             |     |                                  |                 |                   |
|---------------------------------|---------------|---------------|------------|-----------------|-----------------------------|-----|----------------------------------|-----------------|-------------------|
| Gunther, 2017<br>[365]          | Retrospective | 3D            | Other      | Prone           | Numeric margin              | -   | 5-fluorouracil                   | Planned surgery | Out of categories |
| Gurdal, 2018<br>[366]           | Retrospective | IMRT/VMAT     | SIB        | Supine          | Anatomic margin             | Yes | 5-fluorouracil                   | Planned surgery | Out of categories |
| Hernando-Requejo, 2014<br>[367] | Retrospective | IMRT/VMAT     | SIB        | Prone           | No margin                   | Yes | Capecitabine                     | Planned surgery | ≥ 9 weeks         |
| Hong, 2011<br>[368]             | Prospective   | 3D            | Sequential | -               | Numeric and anatomic margin | -   | Capectibanine-irinotecan doublet | Planned surgery | Out of categories |
| Ho-Pun-Cheung, 2007<br>[369]    | Prospective   | 3D            | Sequential | Supine          | Numeric margin              | -   | None                             | Planned surgery | ≥ 9 weeks         |
| Huang, 2014<br>[370]            | Prospective   | IMRT/VMAT     | SIB        | -               | No margin                   | Yes | Capecitabine                     | Planned surgery | < 9 weeks         |
| Ingle, 2023<br>[307]            | In silico     | MRI-guided RT | Sequential | -               | Numeric and anatomic margin | Yes | Capecitabine                     | -               | -                 |
| Jabbour, 2012<br>[371]          | Retrospective | 3D/IMRT/VMAT  | Sequential | Prone or supine | Numeric and anatomic margin | Yes | 5-fluorouracil                   | Planned surgery | -                 |

|                           |               |              |                   |                 |                             |     |                |                  |                   |
|---------------------------|---------------|--------------|-------------------|-----------------|-----------------------------|-----|----------------|------------------|-------------------|
| Jankarashvili, 2019 [372] | Retrospective | IMRT/VMAT    | Sequential or SIB | Supine          | Anatomic margin             | Yes | Capecitabine   | Planned surgery  | < 9 weeks         |
| Karaca, 2021 [373]        | Retrospective | IMRT/VMAT    | Sequential or SIB | Supine          | Numeric and anatomic margin | Yes | 5-fluorouracil | «Watch and Wait» | -                 |
| Kilic, 2015 [374]         | In silico     | 3D/IMRT/VMAT | Sequential        | Prone or supine | Numeric margin              | Yes | -              | -                | -                 |
| Kim, 2016 [375]           | Retrospective | 3D           | Sequential        | Prone           | Anatomic margin             | -   | 5-fluorouracil | Planned surgery  | < 9 weeks         |
| Lee, 2022 [376]           | Prospective   | IMRT/VMAT    | SIB               | Supine          | Numeric and anatomic margin | No  | 5-fluorouracil | Planned surgery  | ≥ 9 weeks         |
| Liu, 2022 [377]           | Prospective   | IMRT/VMAT    | Sequential        | -               | -                           | No  | Capecitabine   | Planned surgery  | Out of categories |
| Lo Greco, 2023 [378]      | Retrospective | 3D/IMRT/VMAT | Sequential        | Prone           | -                           | -   | 5-fluorouracil | Planned surgery  | < 9 weeks         |
| Luna, 2019 [379]          | In silico     | 3D/IMRT/VMAT | Sequential        | Prone           | Numeric and anatomic margin | Yes | Capecitabine   | -                | -                 |

|                        |               |              |                   |        |                             |     |                |                  |                   |
|------------------------|---------------|--------------|-------------------|--------|-----------------------------|-----|----------------|------------------|-------------------|
| Lupattelli, 2023 [380] | Retrospective | IMRT/VMAT    | SIB               | -      | No margin                   | -   | Capecitabine   | «Watch and Wait» | -                 |
| Mazzola, 2016 [381]    | Retrospective | 3D           | Sequential        | Prone  | Anatomic margin             | Yes | 5-fluorouracil | Planned surgery  | Out of categories |
| Mohiuddin, 2006 [382]  | Prospective   | 3D           | Sequential        | -      | Numeric and anatomic margin | -   | 5-fluorouracil | Planned surgery  | < 9 weeks         |
| Movsas, 2006 [383]     | Prospective   | 3D           | Sequential        | Prone  | Numeric margin              | -   | 5-fluorouracil | Planned surgery  | < 9 weeks         |
| Ngan, 2012 [384]       | Prospective   | 3D           | Sequential        | -      | Numeric margin              | -   | None           | Planned surgery  | < 9 weeks         |
| O'Neill, 2008 [385]    | Retrospective | IMRT/VMAT    | Sequential        | Prone  | Numeric margin              | -   | Capecitabine   | Planned surgery  | < 9 weeks         |
| Osti, 2014 [386]       | Prospective   | 3D           | SIB               | -      | Anatomic margin             | No  | Capecitabine   | Planned surgery  | < 9 weeks         |
| Owens, 2020 [387]      | Retrospective | IMRT/VMAT    | SIB               | Supine | Numeric margin              | Yes | Capecitabine   | «Watch and Wait» | 44 days           |
| Parekh, 2013 [388]     | Retrospective | 3D/IMRT/VMAT | Sequential or SIB | Prone  | Numeric margin              | Yes | 5-fluorouracil | Planned surgery  | Out of categories |



|                          |               |                  |            |                 |                             |     |                                  |                 |                   |
|--------------------------|---------------|------------------|------------|-----------------|-----------------------------|-----|----------------------------------|-----------------|-------------------|
| Park, 2011<br>[389]      | Prospective   | 3D               | Sequential | -               | No margin                   | No  | Capecitabine                     | Planned surgery | < 9 weeks         |
| Picardi, 2017<br>[390]   | Prospective   | IMRT/VMAT        | SIB        | Prone           | Numeric and anatomic margin | No  | Capecitabine-oxaliplatin doublet | Planned surgery | < 9 weeks         |
| Radu, 2013<br>[391]      | In silico     | IMRT/VMAT        | Sequential | -               | Numeric margin              | Yes | Capecitabine                     | -               | -                 |
| Samuelian, 2012 [392]    | Retrospective | 3D               | Sequential | Prone or supine | No margin                   | Yes | 5-fluorouracil                   | Planned surgery | < 9 weeks         |
| Schmoll, 2021<br>[393]   | Prospective   | 3D               | Sequential | Prone or supine | -                           | -   | Capecitabine                     | Planned surgery | < 9 weeks         |
| Schrag, 2023<br>[113]    | Prospective   | 3D/IMRT/V<br>MAT | Sequential | Prone or supine | Anatomic margin             | No  | 5-fluorouracil                   | Planned surgery | Out of categories |
| Seierstad, 2009<br>[331] | In silico     | -                | SIB        | -               | No margin                   | Yes | Capecitabine-oxaliplatin doublet | -               | -                 |
| Shepherd, 2020<br>[333]  | In silico     | -                | SIB        | Supine          | -                           | -   | -                                | -               | -                 |
| Spatola, 2019<br>[394]   | Prospective   | 3D/IMRT/V<br>MAT | Other      | Prone           | -                           | Yes | 5-fluorouracil                   | Planned surgery | Out of categories |

|                         |             |               |                   |                 |                 |     |                |                  |                   |
|-------------------------|-------------|---------------|-------------------|-----------------|-----------------|-----|----------------|------------------|-------------------|
| Temelli, 2019 [395]     | In silico   | IMRT/VMAT     | SIB               | Supine          | -               | Yes | -              | -                | -                 |
| Tepper, 2002 [411]      | Prospective | 3D            | Sequential        | -               | Numeric margin  | -   | 5-fluorouracil | Planned surgery  | -                 |
| Tey, 2017 [332]         | Prospective | IMRT/VMAT     | SIB               | Prone           | No margin       | Yes | Capecitabine   | Planned surgery  | Out of categories |
| Tunio, 2010 [396]       | Prospective | 3D            | Sequential        | Prone           | -               | -   | Capecitabine   | Planned surgery  | Out of categories |
| Verweij, 2023 [397]     | Protocol    | MRI-guided RT | Sequential        | -               | No margin       | Yes | None           | «Watch and Wait» | 11 – 13 weeks     |
| Vestermarck, 2012 [398] | Prospective | 3D            | Sequential or SIB | Supine          | -               | Yes | Other-doublet  | Planned surgery  | Out of categories |
| Wawok, 2018 [399]       | Prospective | 3D            | Sequential        | Prone           | Anatomic margin | -   | None           | Planned surgery  | < 9 weeks         |
| Whaley, 2014 [400]      | In silico   | -             | -                 | -               | Anatomic margin | -   | -              | -                | -                 |
| Xu, 2016 [401]          | Prospective | IMRT/VMAT     | SIB               | Prone or supine | No margin       | Yes | Capecitabine   | Planned surgery  | Out of categories |
| Yang, 2015 [403]        | In silico   | IMRT/VMAT     | SIB               | Supine          | No margin       | Yes | -              | -                | -                 |
| Yang, 2019 [402]        | In silico   | 3D            | Sequential        | -               | -               | -   | 5-fluorouracil | -                | -                 |

|                      |               |              |     |        |                 |     |                |                 |           |
|----------------------|---------------|--------------|-----|--------|-----------------|-----|----------------|-----------------|-----------|
| Yang, 2019<br>[404]  | Prospective   | IMRT/VMAT    | SIB | Supine | No margin       | No  | Capecitabine   | Planned surgery | < 9 weeks |
| Zhang, 2019<br>[405] | Protocol      | -            | SIB | Supine | No margin       | Yes | None           | Planned surgery | ≥ 9 weeks |
| Zhao, 2016<br>[406]  | In silico     | 3D/IMRT/VMAT | SIB | Prone  | No margin       | Yes | -              | -               | -         |
| Zhao, 2019<br>[407]  | Prospective   | IMRT/VMAT    | SIB | Supine | No margin       | Yes | Capecitabine   | Planned surgery | < 9 weeks |
| Zhao, 2021<br>[408]  | Retrospective | 3D           | SIB | -      | Anatomic margin | -   | 5-fluorouracil | Planned surgery | -         |

*Table A7.7: List of publications included in the descriptive analysis. 3D: Three-dimensional radiotherapy, cCR: Clinical complete response, CTV: Clinical target volume, IMRT: intensity-modulated radiotherapy, MRI: Magnetic resonance imaging, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

| Institutional duplicates |                            | Publications excluded through quality assessment analysis |                            |
|--------------------------|----------------------------|-----------------------------------------------------------|----------------------------|
| Allal, 2002 [810]        | Freedman, 2007 [811]       | Mohiuddin, 2013 [812]                                     | Avancha, 2022 [813]        |
| Allal, 2005 [814]        | Gani, 2021 [815]           | Musio, 2010 [816]                                         | Avci, 2021 [817]           |
| Alongi, 2017 [818]       | Garcia-Aguilar, 2015 [819] | Myerson, 2001 [820]                                       | Bitterman, 2015 [821]      |
| Alvarez, 2024 [473]      | Garcia-Aguilar, 2015 [822] | Myerson, 2002 [823]                                       | Bountouroglou, 2004 [824]  |
| Azria, 2005 [825]        | Gerard, 2002 [826]         | Park, 2009 [827]                                          | Bruera, 2018 [828]         |
| Bazarbashi, 2016 [829]   | Gerard, 2015 [830]         | Passoni, 2013 [831]                                       | Bujko, 2013 [832]          |
| Bird, 2021 [833]         | Gevaert, 2012 [834]        | Pfeiffer, 2005 [835]                                      | Chan, 2000 [836]           |
| Boldrini, 2018 [837]     | Guillem, 2007 [838]        | Picardi, 2016 [839]                                       | Conroy, 2021 [115]         |
| Bonnen, 2004 [840]       | Habr-Gama, 2006 [841]      | Pietsch, 2007 [842]                                       | D'Amata, 2021 [843]        |
| Bonomo, 2021 [844]       | Habr-Gama, 2009 [845]      | Raso, 2015 [293]                                          | Datta, 2024 [846]          |
| Braendengen, 2008 [847]  | Habr-Gama, 2014 [848]      | Roh, 2009 [101]                                           | De Las Heras, 2013 [849]   |
| Burbach, 2015 [316]      | Habr-Gama, 2019 [850]      | Rouanet, 2002 [851]                                       | Di Tommaso, 2020 [409]     |
| Burbach, 2016 [852]      | Hong, 2015 [853]           | Sanfilippo, 2001 [854]                                    | Dumont, 2018 [855]         |
| Capirci, 2002 [856]      | Ingle, 2022 [857]          | Sao Juliao, 2018 [858]                                    | Garcia-Aguilar, 2011 [859] |
| Caravatta, 2011 [860]    | Janjan, 2000 [861]         | Tuta, 2019 [862]                                          | Habr-Gama, 2013 [863]      |
| Caravatta, 2012 [864]    | Jeong, 2016 [865]          | Urbano, 2006 [866]                                        | Hashimoto, 2023 [867]      |
| Chao, 2005 [868]         | Jin, 2005 [869]            | Valentini, 2001 [870]                                     | Hudson, 2022 [871]         |
| Chau, 2003 [872]         | Kim, 2006 [873]            | Valentini, 2008 [874]                                     | Kennecke, 2012 [875]       |

|                             |                           |                           |                       |
|-----------------------------|---------------------------|---------------------------|-----------------------|
| Chiloiro, 2019<br>[744]     | Kim, 2007 [876]           | Valentini, 2019<br>[289]  | Kim, 2002 [877]       |
| Chiloiro, 2022<br>[878]     | Klautke, 2005<br>[879]    | Velenik, 2020<br>[880]    | Klautke, 2007 [881]   |
| Cilla, 2012 [882]           | Kleijnen, 2019<br>[303]   | Verweij, 2022<br>[165]    | Markovina, 2017 [883] |
| Cilla, 2014 [884]           | Krishnan, 2006<br>[885]   | Vestermark,<br>2008 [886] | Mehta, 2001 [887]     |
| Couwenberg,<br>2020 [164]   | Lee, 2012 [888]           | Wang, 2019<br>[889]       | O'Connell, 2015 [107] |
| Cubillo, 2014<br>[890]      | Lee, 2014 [891]           | White, 2021<br>[802]      | Ofshteyn, 2020 [892]  |
| Czito, 2004 [893]           | Li, 2012 [894]            | Xu, 2014 [232]            | Paszt, 2022 [895]     |
| De Ridder, 2008<br>[797]    | Li, 2021 [896]            | Xu, 2017 [897]            | Rodel, 2000 [898]     |
| Dewdney, 2012<br>[899]      | Loi, 2005 [900]           | Yang, 2014 [241]          | Schou, 2012 [901]     |
| Eijkelenkamp,<br>2021 [585] | Lupattelli, 2006<br>[902] | Zhu, 2013 [903]           | Singh, 2017 [904]     |
| Elashwah, 2023<br>[905]     | Lupattelli, 2017<br>[906] | Zhu, 2014 [907]           | Sirak, 2020 [908]     |
| Engels, 2012 [437]          | Meyer, 2018 [909]         |                           | Wygoda, 2010 [910]    |
| Engineer, 2013<br>[911]     | Mohiuddin, 2000<br>[912]  |                           | Yamashita, 2017 [913] |
| Everaert, 2011<br>[914]     | Mohiuddin, 2000<br>[915]  |                           | Yang, 2017 [916]      |

*Table A7.8: List of publications excluded from the descriptive analysis.*

| Planned surgery publications |                         | Watch and wait publications |
|------------------------------|-------------------------|-----------------------------|
| Allal, 2002 [810]            | Liu, 2022 [377]         | Bitterman, 2015 [821]       |
| Allal, 2005 [814]            | Loi, 2005 [900]         | Habr-Gama, 2006 [841]       |
| Avancha, 2022 [813]          | Markovina, 2017 [883]   | Habr-Gama, 2013 [863]       |
| Avci, 2021 [817]             | Mehta, 2001 [887]       | Habr-Gama, 2014 [848]       |
| Bae, 2017 [342]              | Mohiuddin, 2000 [912]   | Habr-Gama, 2019 [850]       |
| Ballonoff, 2008 [344]        | Mohiuddin, 2000 [915]   | Sao Juliao, 2018 [858]      |
| Bazarbashi, 2016 [829]       | Mohiuddin, 2013 [812]   |                             |
| Braendengen, 2008 [847]      | O'Connell, 2015 [107]   |                             |
| Bruera, 2018 [828]           | Ofshteyn, 2020 [892]    |                             |
| Bujko, 2013 [832]            | Parekh, 2013 [388]      |                             |
| Conroy, 2021 [115]           | Rodel, 2000 [898]       |                             |
| Czito, 2004 [893]            | Roh, 2009 [101]         |                             |
| De Las Heras, 2013 [849]     | Rouanet, 2002 [851]     |                             |
| Dewdney, 2012 [899]          | Samuelian, 2012 [392]   |                             |
| Di Tommaso, 2020 [409]       | Sanfilippo, 2001 [854]  |                             |
| Dumont , 2018 [855]          | Schou, 2012 [901]       |                             |
| Elashwah, 2023 [905]         | Sirak, 2020 [908]       |                             |
| Engineer, 2013 [911]         | Tuta, 2019 [862]        |                             |
| Garcia-Aguilar, 2011 [859]   | Velenik, 2020 [880]     |                             |
| Garcia-Aguilar, 2015 [819]   | Vestermarck, 2008 [886] |                             |
| Guillem, 2007 [838]          | Wawok, 2018 [399]       |                             |
| Jabbour, 2012 [371]          | Wygoda, 2010 [910]      |                             |
| Jeong, 2016 [865]            | Xu, 2014 [917]          |                             |
| Kennecke, 2012 [875]         | Yamashita, 2017 [913]   |                             |
| Kim, 2002 [877]              | Yang, 2017 [916]        |                             |
| Klautke, 2007 [881]          | Zhu, 2013 [903]         |                             |
| Lee, 2014 [891]              |                         |                             |

*Table A7.9: List of publications excluded from the meta-analysis through quality assessment analysis.*

| Author, year            | Publication year | RT Modulation | CTV                           | PTV          | Boost sequence | Boost BED  | Concomitant chemotherapy      | Neo-adjuvant chemotherapy | Surgical delay | pCR reported | Local recurrence rate reported |
|-------------------------|------------------|---------------|-------------------------------|--------------|----------------|------------|-------------------------------|---------------------------|----------------|--------------|--------------------------------|
| Achard, 2021 [335]      | ≥ 2016           | IMRT/VMAT     | GTV + 0-10 mm                 | CTV + 0-5 mm | SIB            | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | OUT            | Yes          | Yes                            |
| Alongi, 2017 [818]      | ≥ 2016           | IMRT/VMAT     | Involved mesorectum           | CTV + 0-5 mm | SIB            | > 74 Gy    | Single-agent fluoropyrimidine | None                      | OUT            | Yes          | No                             |
| Alsuhaibani, 2019 [337] | ≥ 2016           | IMRT/VMAT     | More than involved mesorectum | CTV + 0-5 mm | SIB            | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | OUT            | Yes          | No                             |
|                         | ≥ 2016           | IMRT/VMAT     | More than involved mesorectum | CTV + 0-5 mm | SIB            | > 74 Gy    | Single-agent fluoropyrimidine | None                      | OUT            | Yes          | No                             |
| Badakhshi, 2017 [341]   | ≥ 2016           | 3D            | OUT                           | OUT          | OUT            | OUT        | Single-agent fluoropyrimidine | None                      | OUT            | Yes          | No                             |
| Bertocchi, 2020 [346]   | ≥ 2016           | IMRT/VMAT     | GTV                           | CTV + 0-5 mm | SIB            | 68 - 74 Gy | OUT                           | None                      | OUT            | Yes          | No                             |
|                         | ≥ 2016           | IMRT/VMAT     | GTV                           | CTV + 0-5 mm | SIB            | > 74 Gy    | OUT                           | None                      | OUT            | Yes          | No                             |

|                           |                    |               |                                     |                     |                 |                  |                                  |      |              |     |     |
|---------------------------|--------------------|---------------|-------------------------------------|---------------------|-----------------|------------------|----------------------------------|------|--------------|-----|-----|
| Bonnen, 2004 [840]        | < 2008             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | > 74<br>Gy       | Single-agent<br>fluoropyrimidine | None | OUT          | Yes | No  |
| But-Hadzic,<br>2018 [348] | ≥ 2016             | IMRT/<br>VMAT | GTV + 0-10 mm                       | CTV +<br>5-10<br>mm | SIB             | < 68<br>Gy       | Single-agent<br>fluoropyrimidine | None | < 9<br>weeks | Yes | Yes |
| Capirci, 2009 [349]       | ≥ 2008 -<br>< 2016 | OUT           | GTV + > 10 mm                       | OUT                 | SIB             | > 74<br>Gy       | Single-agent<br>fluoropyrimidine | None | OUT          | Yes | No  |
| Caravatta,<br>2011 [860]  | ≥ 2008 -<br>< 2016 | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB             | > 74<br>Gy       | Other doublet                    | None | < 9<br>weeks | Yes | Yes |
| Caravatta,<br>2012 [864]  | ≥ 2008 -<br>< 2016 | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | Sequen-<br>tial | 68 -<br>74<br>Gy | OUT                              | None | < 9<br>weeks | Yes | Yes |
|                           | ≥ 2008 -<br>< 2016 | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB             | > 74<br>Gy       | OUT                              | None | < 9<br>weeks | Yes | Yes |
|                           | ≥ 2008 -<br>< 2016 | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | OUT             | OUT              | Other doublet                    | None | < 9<br>weeks | Yes | Yes |
| Chao, 2005 [868]          | < 2008             | 3D            | GTV                                 | CTV +<br>5-10<br>mm | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine | None | < 9<br>weeks | Yes | Yes |



|                         |                    |               |                                     |                     |                 |                  |                                  |           |              |     |     |
|-------------------------|--------------------|---------------|-------------------------------------|---------------------|-----------------|------------------|----------------------------------|-----------|--------------|-----|-----|
| Chau, 2003<br>[872]     | < 2008             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine | Induction | < 9<br>weeks | Yes | Yes |
| Chen, 2021<br>[351]     | ≥ 2016             | IMRT/<br>VMAT | OUT                                 | OUT                 | OUT             | OUT              | Single-agent<br>fluoropyrimidine | OUT       | OUT          | Yes | Yes |
| Cubillo, 2014<br>[890]  | ≥ 2008 -<br>< 2016 | OUT           | GTV                                 | CTV +<br>5-10<br>mm | SIB             | > 74<br>Gy       | None                             | None      | < 9<br>weeks | Yes | No  |
| De Bari, 2019<br>[353]  | ≥ 2016             | IMRT/<br>VMAT | More than<br>involved<br>mesorectum | CTV +<br>0-5<br>mm  | SIB             | < 68<br>Gy       | Single-agent<br>fluoropyrimidine | None      | OUT          | Yes | No  |
|                         | ≥ 2016             | IMRT/<br>VMAT | More than<br>involved<br>mesorectum | CTV +<br>0-5<br>mm  | SIB             | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine | None      | OUT          | Yes | No  |
| De Paoli, 2006<br>[354] | < 2008             | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | Sequen-<br>tial | 68 -<br>74<br>Gy | OUT                              | None      | < 9<br>weeks | Yes | No  |
| Engels, 2012<br>[437]   | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV                                 | CTV + ><br>10 mm    | SIB             | < 68<br>Gy       | None                             | None      | < 9<br>weeks | Yes | Yes |
|                         | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV                                 | CTV + ><br>10 mm    | SIB             | > 74<br>Gy       | None                             | None      | < 9<br>weeks | Yes | Yes |
| Engels, 2023<br>[356]   | ≥ 2016             | OUT           | GTV                                 | CTV + ><br>10 mm    | SIB             | < 68<br>Gy       | Single-agent<br>fluoropyrimidine | None      | OUT          | Yes | Yes |

|                       |                 |           |                               |               |            |            |                                      |           |           |     |     |
|-----------------------|-----------------|-----------|-------------------------------|---------------|------------|------------|--------------------------------------|-----------|-----------|-----|-----|
|                       | ≥ 2016          | OUT       | GTV                           | CTV + > 10 mm | SIB        | > 74 Gy    | OUT                                  | None      | OUT       | Yes | Yes |
| Faria, 2014 [357]     | ≥ 2008 - < 2016 | OUT       | GTV                           | CTV + 5-10 mm | SIB        | < 68 Gy    | None                                 | None      | < 9 weeks | Yes | Yes |
| Fiorino, 2018 [358]   | ≥ 2016          | IMRT/VMAT | GTV                           | CTV + 0-5 mm  | SIB        | 68 - 74 Gy | Fluoropyrimidine-oxaliplatin doublet | Induction | OUT       | Yes | No  |
| Freedman, 2007 [811]  | < 2008          | IMRT/VMAT | GTV + 0-10 mm                 | CTV + 5-10 mm | SIB        | > 74 Gy    | Single-agent fluoropyrimidine        | None      | OUT       | Yes | Yes |
| Gasparini, 2012 [361] | ≥ 2008 - < 2016 | OUT       | GTV + > 10 mm                 | OUT           | Sequential | 68 - 74 Gy | Other doublet                        | Induction | < 9 weeks | Yes | Yes |
| Geng, 2021 [362]      | ≥ 2016          | IMRT/VMAT | GTV                           | CTV + 0-5 mm  | SIB        | 68 - 74 Gy | OUT                                  | None      | OUT       | Yes | Yes |
| Gerard, 2003 [363]    | < 2008          | 3D        | GTV + > 10 mm                 | OUT           | Sequential | 68 - 74 Gy | Fluoropyrimidine-oxaliplatin doublet | None      | < 9 weeks | Yes | Yes |
| Guido, 2023 [364]     | ≥ 2016          | IMRT/VMAT | More than involved mesorectum | CTV + 5-10 mm | OUT        | > 74 Gy    | Single-agent fluoropyrimidine        | None      | OUT       | Yes | Yes |

|                        |                    |               |                                     |                     |                 |                  |                                             |      |              |     |     |
|------------------------|--------------------|---------------|-------------------------------------|---------------------|-----------------|------------------|---------------------------------------------|------|--------------|-----|-----|
| Gunther, 2017<br>[365] | ≥ 2016             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | < 68<br>Gy       | Single-agent<br>fluoropyrimidine            | None | OUT          | Yes | No  |
|                        | ≥ 2016             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None | OUT          | Yes | No  |
|                        | ≥ 2016             | IMRT/<br>VMAT | Involved<br>mesorectum              | CTV +<br>5-10<br>mm | SIB             | < 68<br>Gy       | Single-agent<br>fluoropyrimidine            | None | OUT          | Yes | Yes |
|                        | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV                                 | CTV +<br>0-5<br>mm  | SIB             | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None | ≥ 9<br>weeks | Yes | Yes |
|                        | ≥ 2008 -<br>< 2016 | 3D            | More than<br>involved<br>mesorectum | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Other doublet                               | None | OUT          | Yes | Yes |
|                        | ≥ 2008 -<br>< 2016 | OUT           | More than<br>involved<br>mesorectum | CTV +<br>0-5<br>mm  | Sequen-<br>tial | 68 -<br>74<br>Gy | Fluoropyrimidine-<br>oxaliplatin<br>doublet | None | < 9<br>weeks | Yes | Yes |
|                        | < 2008             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | > 74<br>Gy       | None                                        | None | ≥ 9<br>weeks | Yes | Yes |
|                        | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV                                 | CTV +<br>0-5<br>mm  | SIB             | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None | < 9<br>weeks | Yes | Yes |
| Janjan, 2000<br>[861]  | < 2008             | 3D            | GTV + > 10 mm                       | OUT                 | Sequen-<br>tial | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None | OUT          | Yes | No  |

|                           |                 |            |                               |               |            |            |                               |      |           |     |     |
|---------------------------|-----------------|------------|-------------------------------|---------------|------------|------------|-------------------------------|------|-----------|-----|-----|
| Jankarashvili, 2019 [372] | ≥ 2016          | IMRT/ VMAT | OUT                           | CTV + 0-5 mm  | Sequencial | 68 - 74 Gy | Single-agent fluoropyrimidine | None | < 9 weeks | Yes | No  |
|                           | ≥ 2016          | IMRT/ VMAT | OUT                           | CTV + 0-5 mm  | SIB        | > 74 Gy    | Single-agent fluoropyrimidine | None | < 9 weeks | Yes | No  |
| Jin, 2005 [869]           | < 2008          | 3D         | GTV                           | CTV + > 10 mm | Sequencial | 68 - 74 Gy | Single-agent fluoropyrimidine | None | < 9 weeks | Yes | No  |
| Kim, 2016 [375]           | ≥ 2016          | 3D         | Involved mesorectum           | OUT           | Sequencial | 68 - 74 Gy | OUT                           | None | < 9 weeks | Yes | Yes |
| Krishnan, 2006 [885]      | < 2008          | 3D         | More than involved mesorectum | OUT           | Sequencial | > 74 Gy    | Other doublet                 | None | OUT       | Yes | Yes |
| Lee, 2012 [888]           | ≥ 2008 - < 2016 | 3D         | GTV                           | CTV + > 10 mm | SIB        | 68 - 74 Gy | Single-agent fluoropyrimidine | None | < 9 weeks | Yes | No  |
| Lee, 2022 [376]           | ≥ 2016          | IMRT/ VMAT | More than involved mesorectum | CTV + 5-10 mm | SIB        | > 74 Gy    | Single-agent fluoropyrimidine | None | ≥ 9 weeks | Yes | Yes |
| Li, 2012 [894]            | ≥ 2008 - < 2016 | IMRT/ VMAT | Involved mesorectum           | CTV + 5-10 mm | SIB        | 68 - 74 Gy | Single-agent fluoropyrimidine | None | < 9 weeks | Yes | Yes |

|                           |        |     |                                     |                     |                |                  |                                             |                                |              |     |     |
|---------------------------|--------|-----|-------------------------------------|---------------------|----------------|------------------|---------------------------------------------|--------------------------------|--------------|-----|-----|
| Lo Greco, 2023<br>[378]   | ≥ 2016 | OUT | OUT                                 | OUT                 | OUT            | OUT              | OUT                                         | Induction and<br>consolidation | < 9<br>weeks | Yes | Yes |
| Lupattelli,<br>2006 [902] | < 2008 | 3D  | More than<br>involved<br>mesorectum | CTV +<br>0-5<br>mm  | Sequen<br>tial | 68 -<br>74<br>Gy | Fluoropyrimidine-<br>oxaliplatin<br>doublet | None                           | < 9<br>weeks | Yes | Yes |
| Mazzola, 2016<br>[381]    | ≥ 2016 | 3D  | Involved<br>mesorectum              | CTV +<br>5-10<br>mm | Sequen<br>tial | < 68<br>Gy       | Single-agent<br>fluoropyrimidine            | None                           | OUT          | Yes | No  |
|                           | ≥ 2016 | 3D  | Involved<br>mesorectum              | CTV +<br>5-10<br>mm | Sequen<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None                           | OUT          | Yes | No  |
| Meyer, 2017<br>[909]      | ≥ 2016 | OUT | Involved<br>mesorectum              | CTV +<br>5-10<br>mm | Sequen<br>tial | 68 -<br>74<br>Gy | Other doublet                               | Consolidation                  | < 9<br>weeks | Yes | Yes |
| Mohiuddin,<br>2006 [382]  | < 2008 | 3D  | More than<br>involved<br>mesorectum | OUT                 | Sequen<br>tial | OUT              | Single-agent<br>fluoropyrimidine            | None                           | < 9<br>weeks | Yes | No  |
|                           | < 2008 | 3D  | More than<br>involved<br>mesorectum | OUT                 | Sequen<br>tial | 68 -<br>74<br>Gy | Other doublet                               | None                           | < 9<br>weeks | Yes | No  |
| Movsas, 2006<br>[383]     | < 2008 | 3D  | GTV + > 10 mm                       | OUT                 | Sequen<br>tial | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None                           | < 9<br>weeks | Yes | Yes |

|     |                        |                    |               |                        |                     |                 |                  |                                             |           |              |     |     |
|-----|------------------------|--------------------|---------------|------------------------|---------------------|-----------------|------------------|---------------------------------------------|-----------|--------------|-----|-----|
| 553 | Musio, 2010<br>[816]   | ≥ 2008 -<br>< 2016 | 3D            | GTV + > 10 mm          | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None      | ≥ 9<br>weeks | Yes | No  |
|     |                        | ≥ 2008 -<br>< 2016 | 3D            | GTV + > 10 mm          | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Fluoropyrimidine-<br>oxaliplatin<br>doublet | None      | ≥ 9<br>weeks | Yes | No  |
|     | Myerson, 2001<br>[820] | < 2008             | 3D            | Involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB             | OUT              | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | No  |
|     | Ngan, 2012<br>[384]    | ≥ 2008 -<br>< 2016 | 3D            | GTV + > 10 mm          | OUT                 | OUT             | < 68<br>Gy       | None                                        | None      | < 9<br>weeks | Yes | Yes |
|     |                        | ≥ 2008 -<br>< 2016 | 3D            | GTV + > 10 mm          | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | Yes |
|     | O'Neill, 2008<br>[385] | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV + > 10 mm          | OUT                 | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | Induction | < 9<br>weeks | Yes | No  |
|     | Osti, 2014<br>[386]    | ≥ 2008 -<br>< 2016 | 3D            | Involved<br>mesorectum | CTV + ><br>10 mm    | SIB             | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | Yes |
|     | Park, 2009<br>[827]    | ≥ 2008 -<br>< 2016 | 3D            | OUT                    | OUT                 | SIB             | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None      | OUT          | Yes | Yes |
|     | Park, 2011<br>[389]    | ≥ 2008 -<br>< 2016 | 3D            | GTV                    | CTV + ><br>10 mm    | Sequen-<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | Yes |

|                        |                    |               |                                     |                     |                |                  |                                             |           |              |     |     |
|------------------------|--------------------|---------------|-------------------------------------|---------------------|----------------|------------------|---------------------------------------------|-----------|--------------|-----|-----|
| Passoni, 2013<br>[831] | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | GTV                                 | CTV +<br>0-5<br>mm  | SIB            | 68 -<br>74<br>Gy | Fluoropyrimidine-<br>oxaliplatin<br>doublet | None      | ≥ 9<br>weeks | Yes | No  |
| Picardi, 2016<br>[839] | ≥ 2016             | 3D            | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB            | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | No  |
| Picardi, 2017<br>[390] | ≥ 2016             | IMRT/<br>VMAT | More than<br>involved<br>mesorectum | CTV + ><br>10 mm    | SIB            | > 74<br>Gy       | Other doublet                               | None      | < 9<br>weeks | Yes | Yes |
| Schmoll, 2020<br>[393] | ≥ 2016             | 3D            | OUT                                 | OUT                 | OUT            | OUT              | Single-agent<br>fluoropyrimidine            | None      | < 9<br>weeks | Yes | Yes |
|                        | ≥ 2016             | 3D            | OUT                                 | OUT                 | OUT            | OUT              | Fluoropyrimidine-<br>oxaliplatin<br>doublet | None      | < 9<br>weeks | Yes | Yes |
| Schrag, 2023<br>[113]  | ≥ 2016             | OUT           | OUT                                 | CTV + ><br>10 mm    | Sequen<br>tial | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None      | OUT          | Yes | Yes |
| Spatola, 2019<br>[394] | ≥ 2016             | OUT           | OUT                                 | CTV + ><br>10 mm    | OUT            | > 74<br>Gy       | Single-agent<br>fluoropyrimidine            | Induction | OUT          | Yes | Yes |
| Tey, 2017<br>[332]     | ≥ 2016             | IMRT/<br>VMAT | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB            | 68 -<br>74<br>Gy | Single-agent<br>fluoropyrimidine            | None      | OUT          | Yes | Yes |
| Tunio, 2010<br>[396]   | ≥ 2008 -<br>< 2016 | 3D            | OUT                                 | OUT                 | OUT            | OUT              | Single-agent<br>fluoropyrimidine            | None      | OUT          | Yes | No  |

|     |                         |                 |           |                     |               |     |            |                                      |                             |           |     |    |
|-----|-------------------------|-----------------|-----------|---------------------|---------------|-----|------------|--------------------------------------|-----------------------------|-----------|-----|----|
| 555 | Valentini, 2019 [289]   | ≥ 2016          | OUT       | Involved mesorectum | CTV + 5-10 mm | OUT | > 74 Gy    | Single-agent fluoropyrimidine        | None                        | < 9 weeks | Yes | No |
|     |                         | ≥ 2016          | OUT       | Involved mesorectum | CTV + 5-10 mm | OUT | 68 - 74 Gy | Fluoropyrimidine-oxaliplatin doublet | None                        | < 9 weeks | Yes | No |
|     | Vestermarck, 2012 [398] | ≥ 2008 - < 2016 | 3D        | OUT                 | CTV + 5-10 mm | OUT | > 74 Gy    | Fluoropyrimidine-oxaliplatin doublet | Induction and consolidation | OUT       | Yes | No |
|     | Wang, 2019 [889]        | ≥ 2016          | IMRT/VMAT | OUT                 | OUT           | SIB | 68 - 74 Gy | Fluoropyrimidine-oxaliplatin doublet | Consolidation               | < 9 weeks | Yes | No |
|     |                         | ≥ 2016          | IMRT/VMAT | OUT                 | OUT           | SIB | > 74 Gy    | Fluoropyrimidine-oxaliplatin doublet | Consolidation               | < 9 weeks | Yes | No |
|     | Xu, 2017 [897]          | ≥ 2016          | OUT       | OUT                 | CTV + > 10 mm | OUT | 68 - 74 Gy | Fluoropyrimidine-oxaliplatin doublet | Consolidation               | OUT       | Yes | No |
|     | Xu, 2016 [401]          | ≥ 2016          | IMRT/VMAT | GTV                 | CTV + 5-10 mm | SIB | > 74 Gy    | Single-agent fluoropyrimidine        | None                        | OUT       | Yes | No |
|     | Yang, 2019 [404]        | ≥ 2016          | IMRT/VMAT | GTV                 | CTV + 5-10 mm | SIB | > 74 Gy    | Single-agent fluoropyrimidine        | Consolidation               | < 9 weeks | Yes | No |



|                     |                    |               |                                     |                     |     |                  |                                             |               |              |     |     |
|---------------------|--------------------|---------------|-------------------------------------|---------------------|-----|------------------|---------------------------------------------|---------------|--------------|-----|-----|
| Zhao, 2019<br>[407] | ≥ 2016             | IMRT/<br>VMAT | GTV                                 | CTV +<br>0-5<br>mm  | SIB | > 74<br>Gy       | OUT                                         | None          | < 9<br>weeks | Yes | Yes |
| Zhu, 2014<br>[907]  | ≥ 2008 -<br>< 2016 | IMRT/<br>VMAT | More than<br>involved<br>mesorectum | CTV +<br>5-10<br>mm | SIB | 68 -<br>74<br>Gy | Fluoropyrimidine-<br>oxaliplatin<br>doublet | Consolidation | OUT          | Yes | Yes |

*Table A7.10: List of included publications in the meta-analysis for planned surgery cohorts. 3D: Three-dimensional radiotherapy, BED: Biological equivalent dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, OUT: Out of category, pCR: Pathological complete response, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

| Author, year               | Publication year | RT Modulation | CTV                 | PTV           | Boost sequence | Boost BED  | Concomitant chemotherapy      | Neo-adjuvant chemotherapy | cCR reported | Local recurrence rate reported |
|----------------------------|------------------|---------------|---------------------|---------------|----------------|------------|-------------------------------|---------------------------|--------------|--------------------------------|
| Boeke, 2022 [347]          | ≥ 2016           | OUT           | GTV                 | CTV + 5-10 mm | OUT            | > 74 Gy    | None                          | None                      | Yes          | Yes                            |
| Chiloiro, 2019 [744]       | ≥ 2016           | OUT           | Involved mesorectum | CTV + 0-5 mm  | SIB            | > 74 Gy    | OUT                           | OUT                       | Yes          | No                             |
| Couwenberg, 2020 [164]     | ≥ 2016           | IMRT/V MAT    | GTV                 | CTV + > 10 mm | Sequential     | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | Yes          | Yes                            |
|                            | ≥ 2016           | IMRT/V MAT    | GTV                 | CTV + > 10 mm | Sequential     | > 74 Gy    | Single-agent fluoropyrimidine | None                      | Yes          | Yes                            |
| Garant, 2022 [360]         | ≥ 2016           | 3D            | GTV + > 10 mm       | CTV + 5-10 mm | Sequential     | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | Yes          | No                             |
| Garcia-Aguilar, 2022 [103] | ≥ 2016           | OUT           | OUT                 | CTV + > 10 mm | OUT            | OUT        | Single-agent fluoropyrimidine | Induction                 | Yes          | Yes                            |
|                            | ≥ 2016           | OUT           | OUT                 | CTV + > 10 mm | OUT            | OUT        | Single-agent fluoropyrimidine | Consolidation             | Yes          | Yes                            |
| Gerard, 2023 [121]         | ≥ 2016           | OUT           | GTV                 | CTV + > 10 mm | Sequential     | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | Yes          | Yes                            |
| Lupattelli, 2023 [380]     | ≥ 2016           | IMRT/V MAT    | GTV                 | OUT           | SIB            | > 74 Gy    | Single-agent fluoropyrimidine | None                      | Yes          | Yes                            |
| Owens, 2020 [387]          | ≥ 2016           | IMRT/V MAT    | GTV + 0-10 mm       | CTV + > 10 mm | SIB            | 68 - 74 Gy | Single-agent fluoropyrimidine | None                      | Yes          | No                             |

*Table A7.11: : List of included publications in the meta-analysis for «Watch and Wait» cohorts. 3D: Three-dimensional radiotherapy, BED: Biological equivalent dose, cCR: Clinical complete response, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, OUT: Out of category, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

| $I^2$  | $\tau^2$ | Q test of heterogeneity<br>p-value |
|--------|----------|------------------------------------|
| < 25%  | <0.1     |                                    |
| < 50%  | <0.3     | >0.05                              |
| < 75%  | <1       | <0.05                              |
| < 100% | >1       | <0.01                              |

| Meta-analysis of<br>pathological complete<br>response<br>in planned surgery<br>publications |  | $I^2$      | $\tau^2$    | Q test of<br>heterogeneity p-<br>value |
|---------------------------------------------------------------------------------------------|--|------------|-------------|----------------------------------------|
| <b>Publication year</b>                                                                     |  | <b>74%</b> | <b>0.25</b> | <b>&lt; 0.01</b>                       |
| < 2008                                                                                      |  | 63%        | 0.30        | < 0.01                                 |
| ≥ 2008 - < 2016                                                                             |  | 72%        | 0.25        | < 0.01                                 |
| ≥ 2016                                                                                      |  | 79%        | 0.23        | < 0.01                                 |
| <b>RT Modulation</b>                                                                        |  | <b>69%</b> | <b>0.21</b> | <b>&lt; 0.01</b>                       |
| 3D                                                                                          |  | 73%        | 0.22        | < 0.01                                 |
| IMRT/VMAT                                                                                   |  | 43%        | 0.12        | 0.01                                   |
| <b>CTV</b>                                                                                  |  | <b>66%</b> | <b>0.19</b> | <b>&lt; 0.01</b>                       |
| GTV                                                                                         |  | 57%        | 0.19        | < 0.01                                 |
| GTV + 0-10 mm                                                                               |  | 0%         | 0.19        | 1.00                                   |
| GTV + > 10 mm                                                                               |  | 80%        | 0.19        | < 0.01                                 |
| Involved mesorectum                                                                         |  | 77%        | 0.19        | < 0.01                                 |
| Larger than involved<br>mesorectum                                                          |  | 47%        | 0.19        | 0.02                                   |

|                                      |            |             |                  |
|--------------------------------------|------------|-------------|------------------|
| <b>PTV</b>                           | <b>56%</b> | <b>0.14</b> | <b>&lt; 0.01</b> |
| CTV + 0-5 mm                         | 55%        | 0.13        | < 0.01           |
| CTV + 5-10 mm                        | 48%        | 0.15        | < 0.01           |
| CTV + > 10 mm                        | 38%        | 0.04        | 0.1              |
| <b>Boost sequence</b>                | <b>55%</b> | <b>0.17</b> | <b>&lt; 0.01</b> |
| Sequential                           | 59%        | 0.16        | < 0.01           |
| SIB                                  | 49%        | 0.14        | < 0.01           |
| <b>Boost BED</b>                     | <b>59%</b> | <b>0.26</b> | <b>&lt; 0.01</b> |
| < 68 Gy                              | 77%        | 0.81        | < 0.01           |
| 68 - 74 Gy                           | 50%        | 0.09        | < 0.01           |
| > 74 Gy                              | 50%        | 0.20        | < 0.01           |
| <b>Concomitant chemotherapy</b>      | <b>70%</b> | <b>0.29</b> | <b>&lt; 0.01</b> |
| None                                 | 80%        | 1.11        | < 0.01           |
| Single-agent fluoropyrimidine        | 68%        | 0.19        | < 0.01           |
| Fluoropyrimidine-oxaliplatin doublet | 70%        | 0.09        | < 0.01           |
| Other doublet                        | 0%         | 0.00        | 0.51             |
| <b>Neo-adjuvant chemotherapy</b>     | <b>74%</b> | <b>0.26</b> | <b>&lt; 0.01</b> |
| None                                 | 74%        | 0.20        | < 0.01           |
| Induction                            | 60%        | 0.20        | 0.04             |
| Consolidation                        | 0%         | 0.20        | 0.46             |

|                            |     |            |             |                  |
|----------------------------|-----|------------|-------------|------------------|
| Induction<br>consolidation | and | 80%        | 0.20        | 0.03             |
| <b>Surgical delay</b>      |     | <b>81%</b> | <b>0.39</b> | <b>&lt; 0.01</b> |
| < 9 weeks                  |     | 82%        | 0.39        | < 0.01           |
| ≥ 9 weeks                  |     | 71%        | 0.39        | < 0.01           |

*Table A7.12: Heterogeneity analysis of the pathological complete response meta-analysis of planned surgery publications. 3D: Three-dimensional radiotherapy, BED: Biological equivalent dose, CTV: Clinical target volume, GTV: Gross tumour volume, IMRT: intensity-modulated radiotherapy, PTV: Planning target volume, RT: Radiotherapy, SIB: simultaneous integrated boost, VMAT: Volumetric-modulated arc radiotherapy.*

## **7.8. Appendix 8: Markerless liver online adaptive stereotactic radiotherapy: Feasibility analysis**

### **7.8.1. Selection of the best ETHOS 2.0 propagation strategies.**

We included 10 cone-beam computed tomographies (CBCT) from 5 patients of the cohort with markers for this preliminary analysis: for each patient, we selected the two CBCTs for which the highest and the lowest length of the vector between the  $GTV_{\text{Propagated}}$  and the  $GTV_{\text{Groundtruth}}$  ( $L_{\text{CoM}}$ ) was obtained when using a structure-guided deformable image registration (DIR) with deformable GTV propagation. We evaluated on each of these CBCTs the six new propagation strategies provided by the manufacturer: structure-guided DIR with either deformable GTV propagation, slice-wise rigid propagation, and rigid propagation; image-guided DIR with either deformable GTV propagation, slice-wise rigid propagation, and rigid propagation.  $L_{\text{CoM}}$  and volume difference between  $GTV_{\text{Propagated}}$  and  $GTV_{\text{Groundtruth}}$  were reported (Figure A8.1).  $L_{\text{CoM}}$  was significantly lower for image-guided DIR than for structure-guided DIR. Furthermore, volume conservation was preserved when using rigid GTV propagation, whereas some important variations existed for the other propagation strategies. Based on these preliminary results, the image-guided DIR with rigid GTV propagation strategy was selected as the best strategy.

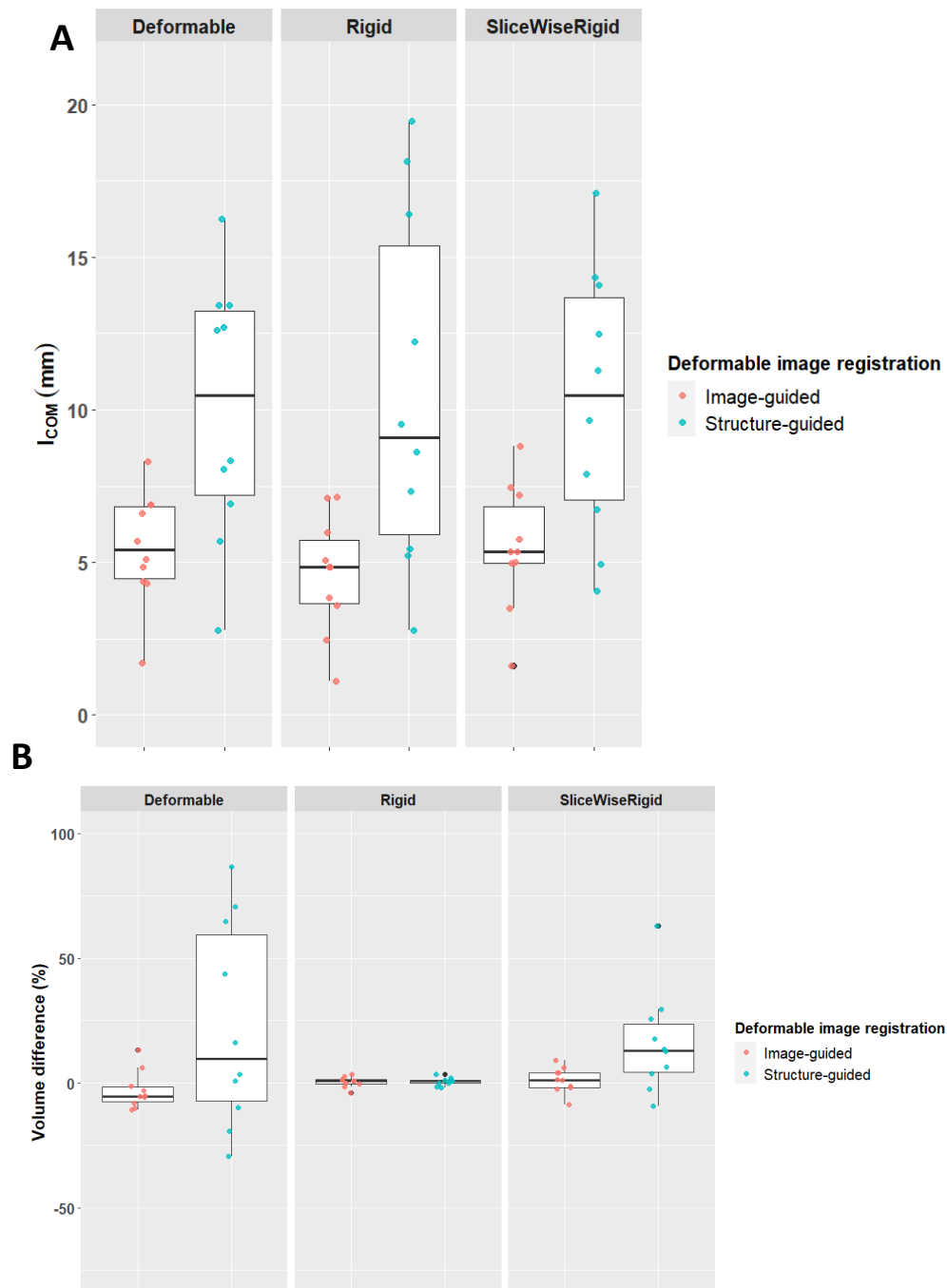




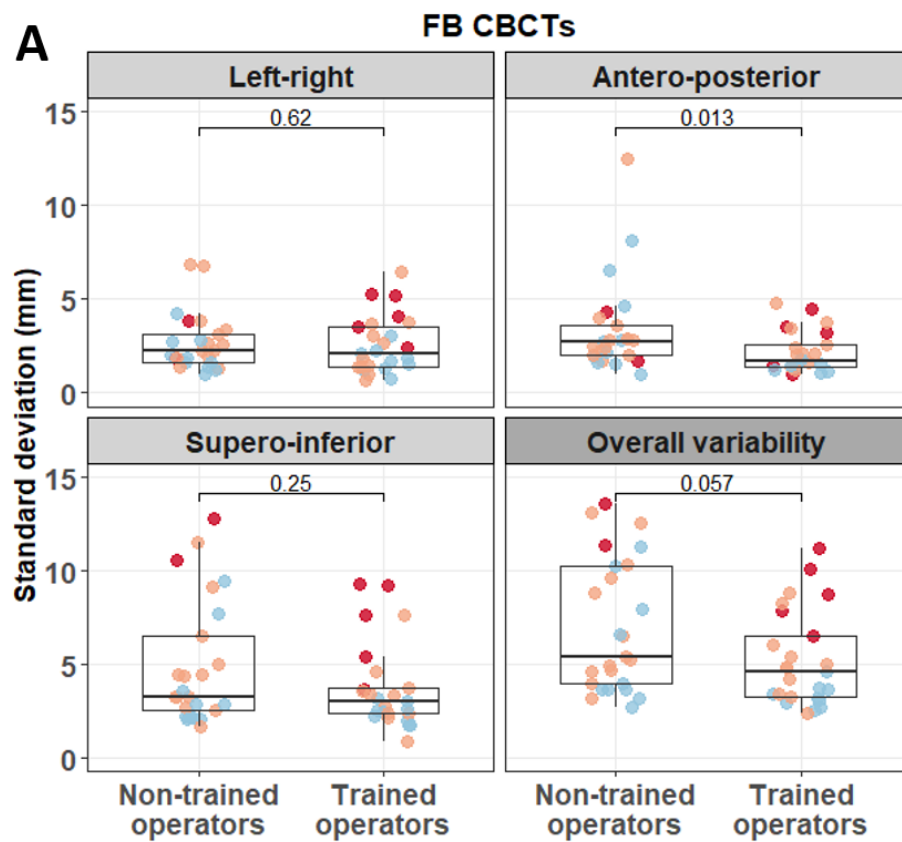
Figure A8.1: Accuracy of target volume propagation using the new CT-STAR propagation strategies. The vector length between the  $GTV_{Propagated}$  and the  $GTV_{Groundtruth}$  ( $l_{CoM}$ ) was significantly lower ( $p < 0.001$ ) when using image-guided deformable registration compared to structure-guided deformable registration (A). The GTV volume was conserved when using rigid propagation whereas, this was not the case for deformable propagation and slice-wise rigid propagation (B). Wilcoxon Signed-Rank Test,  $p < 0.05$  is considered significant. CT-STAR: Computed tomography-guided stereotactic adaptive radiotherapy, GTV: Gross tumour volume.

### 7.8.2. Values of the errors used for the PTV margin calculation.

|                | Left-right  | Antero-posterior | Supero-inferior |
|----------------|-------------|------------------|-----------------|
| Tumour motion  |             |                  |                 |
| $\Sigma$       | 0.5         | 0.5              | 0.5             |
| $\sigma$       | 0.22 - 3.31 | 0.36 – 2.65      | 0.94 – 11.57    |
| Baseline shift |             |                  |                 |
| $\Sigma$       | 0.6         | 1.2              | 0.8             |
| $\sigma$       | 1.4         | 1.8              | 1.6             |
| Delineation    |             |                  |                 |
| $\Sigma$       | 1           | 1                | 1               |
| Penumbra       | 2.9         | 2.9              | 2.9             |

Table A8.1: Values of the systematic ( $\Sigma$ ) and random ( $\sigma$ ) errors used for the PTV margin calculation in the three dimensions. Random errors of the tumour motion were computed individually according to the tumour motion amplitudes measured in the 4D-planning CT along each axis [173].

## 7.9. Appendix 9: Mechanically assisted non-invasive ventilation for liver SABR: Improve CBCT, treat more accurately.



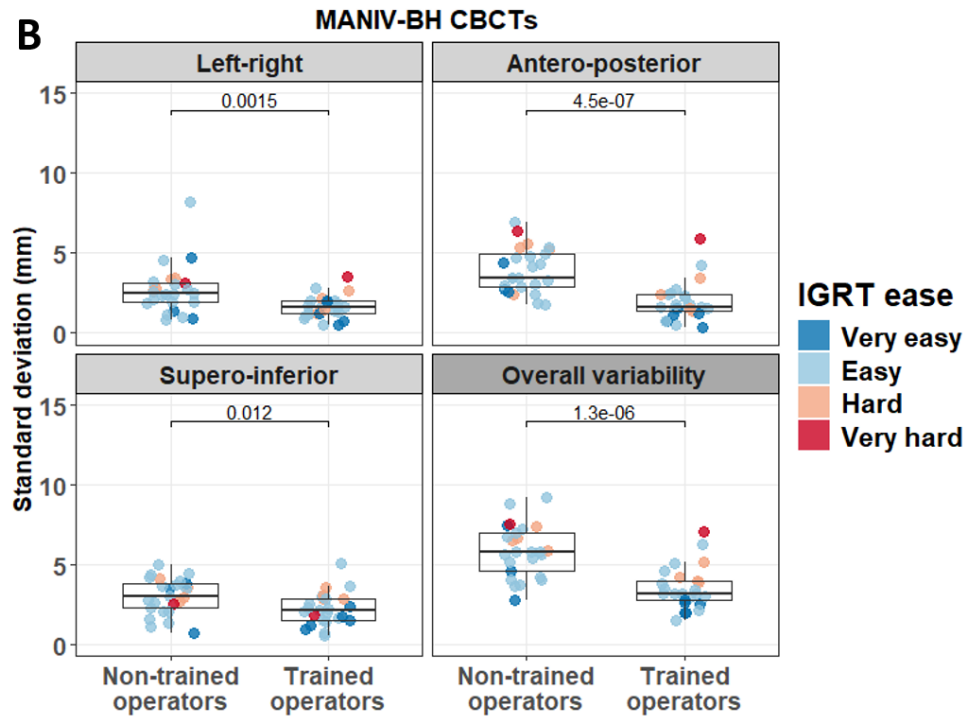


Figure A9.1: Comparison of the image-guided RT variability between non-trained operators and trained operators for (A) free-breathing and (B) MANIV-BH CBCTs. The colours of the points, each representing a CBCT, were determined based on the most frequent image-guided RT ease score reported by the operators. CBCT: Cone-beam computed tomography, FB: Free breathing, IGRT: Image-guided radiotherapy, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold.

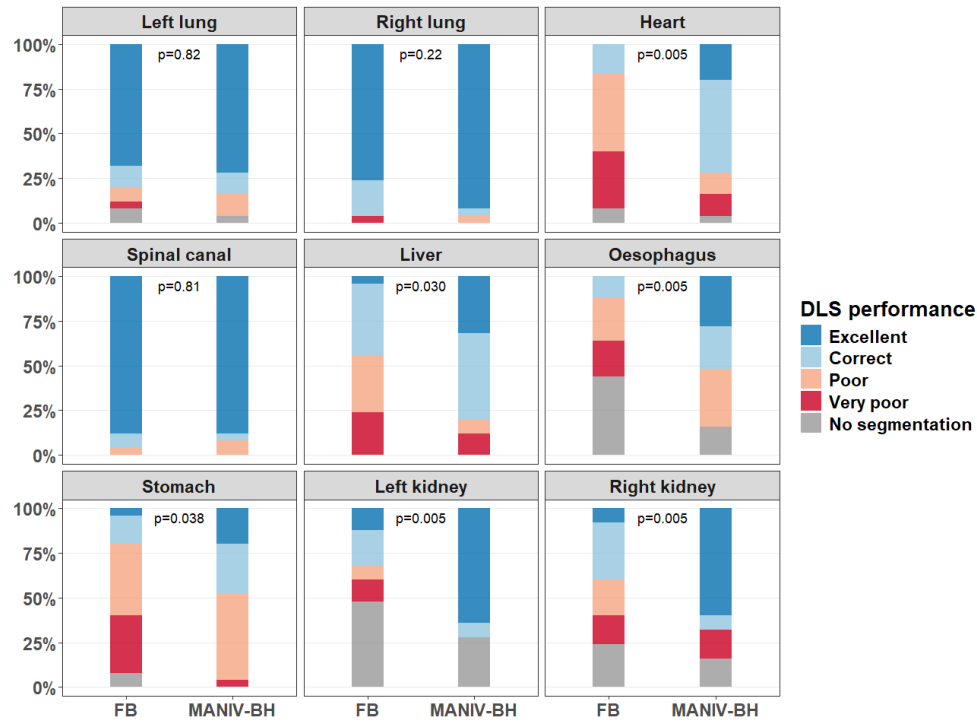


Figure A9.2: Per organ comparison between free-breathing and MANIV-BH CBCTs of the auto-segmentation performance of organs-at-risk using a deep learning algorithm. Statistical comparison was performed using chi-square test with Benjamini-Hochberg adjustment for multiple comparisons. CBCT: Cone-beam computed tomography, FB: Free breathing, MANIV-BH: Mechanically-assisted non-invasive ventilation for breath hold.

| Free-breathing patients included in the “Target volume propagation accuracy” analysis |                    |
|---------------------------------------------------------------------------------------|--------------------|
| <b>N</b>                                                                              | 21                 |
| <b>Gender</b>                                                                         |                    |
| Male                                                                                  | 14                 |
| Female                                                                                | 7                  |
| <b>Age (median [interquartile range])</b>                                             | 65 (59 -75)        |
| <b>Primary disease</b>                                                                |                    |
| Colorectal adenocarcinoma                                                             | 14                 |
| Hepatocarcinoma                                                                       | 4                  |
| Ovary cancer                                                                          | 1                  |
| Non-small cell lung cancer                                                            | 1                  |
| Breast cancer                                                                         | 1                  |
| <b>Tumour location*</b>                                                               |                    |
| Segment I                                                                             | 5                  |
| Segment II                                                                            | 3                  |
| Segment III                                                                           | 1                  |
| Segment IV                                                                            | 5                  |
| Segment V                                                                             | 1                  |
| Segment VI                                                                            | 1                  |
| Segment VII                                                                           | 6                  |
| Segment VIII                                                                          | 8                  |
| <b>GTV volume (cm<sup>3</sup>, median [interquartile range])</b>                      | 25.9 (11.1 – 44.2) |

Table A9.1: Characteristics of the patients included in the “Target volume propagation accuracy” analysis. \*Some patients had tumour involving multiple hepatic segments. GTV: Gross tumour volume.

## 7.10. Appendix 10: Radiotherapy in MSS colorectal cancer: immune impact of the dose and the anatomical site

| Structures            | Objectives                           |
|-----------------------|--------------------------------------|
| <b>Target volumes</b> |                                      |
| GTV_Mod               | D0.1cc < 2880cGy<br>D98% > 2400 cGy  |
| GTV_Low               | D0.1cc < 600 cGy<br>D100% > 300 cGy  |
| GTV_Out               | Dmean < 50 cGy                       |
| PTV_Mod               | D0.1cc < 2880cGy<br>D95% > 2400 cGy  |
| PTV_Low               | D0.1cc < 600 cGy<br>D100% > 300 cGy  |
| PTV_Out               | Dmean < 50 cGy                       |
| <b>Organs at risk</b> |                                      |
| Bladder               | D0.1cc < 2820cGy                     |
| BronchialTree         | D0.1cc < 3000cGy                     |
| ChestWall             | D30cc < 3000cGy                      |
| Colon                 | D0.1cc < 2820cGy                     |
| Duodenum              | D0.1cc < 2520cGy<br>D10cc < 1140cGy  |
| Esophagus             | D0.1cc < 2520cGy                     |
| GreatVessel_PRV*      | ALARA                                |
| Heart                 | ALARA                                |
| Kidney_Left           | Dmean < 850cGy                       |
| Kidney_Right          | Dmean < 850cGy                       |
| Liver_Hilum_PRV       | ALARA                                |
| Liver-PTV             | D>700cc < 1500cGy<br>Dmean < 1300cGy |
| Lungs                 | V2000cGy < 10%<br>Dmean < 800cGy     |
| Lung_Left_Hilum_PRV   | ALARA                                |

|                      |                                     |
|----------------------|-------------------------------------|
| Lung_Right_Hilum_PRV | ALARA                               |
| Rectum               | D0.1cc < 2820cGy                    |
| Skin                 | D10cc < 3000cGy                     |
| SmallBowel           | D0.1cc < 2520cGy<br>D5cc < 1770cGy  |
| SpinalCanal          | D0.1cc < 2030cGy                    |
| Spleen               | ALARA                               |
| Stomach              | D0.1cc < 2220cGy<br>D10cc < 1650cGy |
| Trachea              | D0.1cc < 3000cGy                    |

*Table A10.1: Radiotherapy planning objectives. For PRV structures, an isotropic 5-mm margin around the organ was used. \*Vascular structures included in GreatVessels\_PRV: Aorta, Common iliac vessels, Coronary artery, Hepatic veins, Inferior vena cava, Portal vein, Pulmonary arteries and veins. ALARA: As low as reasonably achievable, GTV: Gross tumour volume, PRV: Planning organ at risk volume, PTV: Planning target volume.*

| Patients | Anatomical site | RT group                    | Gender | Age category | Tumour location    | KRAS mutation | BRAF mutation | Preoperative treatment                                |
|----------|-----------------|-----------------------------|--------|--------------|--------------------|---------------|---------------|-------------------------------------------------------|
| P01      | Liver           | High-RT<br>Low-RT<br>Out-RT | M      | 60 - 75      | Rectum             | No            | No            | 5-Fluorouracile, irinotecan, cetuximab                |
| P01*     |                 | No-RT                       | M      | > 75         | Rectum             | No            | No            | <b>5-Fluorouracile, irinotecan, rectum RT</b>         |
| P02      | Liver           | High-RT<br>Out-RT<br>No-RT  | M      | 60 - 75      | Right colon        | No            | No            | 5-Fluorouracile, oxaliplatin, irinotecan, panitumumab |
| P03      |                 | High-RT<br>Low-RT<br>Out-RT | F      | 60 - 75      | Left colon         | Yes           | No            | 5-Fluorouracile, oxaliplatin, bevacizumab             |
| P03*     |                 | No-RT                       | F      | 60 - 75      | <b>Right colon</b> | Yes           | No            | 5-Fluorouracile, oxaliplatin, bevacizumab             |
| P04      | Liver           | High-RT<br>Out-RT           | F      | 60 - 75      | Right colon        | Yes           | No            | 5-Fluorouracile, oxaliplatin                          |



|      |       |                   |   |                |                   |           |         |                                                       |
|------|-------|-------------------|---|----------------|-------------------|-----------|---------|-------------------------------------------------------|
| P04* |       | No-RT             | F | < 60           | Right colon       | Yes       | No      | <b>5-Fluorouracile, oxaliplatin, irinotecan</b>       |
| P05  | Liver | High-RT<br>No-RT  | M | 60 - 75        | Left colon        | No        | No      | 5-Fluorouracile, oxaliplatin, cetuximab               |
| P06  | Lung  | High-RT           | M | 60 - 75        | Right colon       | Unknown   | Unknown | 5-Fluorouracile, oxaliplatin                          |
| P06* |       | No-RT             | M | 60 - 75        | <b>Rectum</b>     | Unknown   | Unknown | <b>5-Fluorouracile, oxaliplatin, rectum RT</b>        |
| P07  | Liver | High-RT<br>No-RT  | F | 60 - 75        | Left colon        | No        | No      | Intra-peritoneal chemo-hyperthermia                   |
| P08  | Liver | High-RT           | F | < 60           | Rectum            | No        | No      | 5-Fluorouracile, oxaliplatin, irinotecan, panitumumab |
| P08* |       | No-RT             | F | < 60           | Rectum            | No        | No      | <b>5-Fluorouracile, oxaliplatin, panitumumab</b>      |
| P09  | Lung  | High-RT<br>Out-RT | M | 60 - 75        | Rectum            | Yes       | No      | None                                                  |
| P09* |       | No-RT             | M | 60 - 75        | <b>Left colon</b> | Yes       | No      | None                                                  |
| P10  | Liver | High-RT           | F | < 60           | Rectum            | Yes       | No      | 5-Fluorouracile, oxaliplatin, irinotecan              |
| P10* |       | No-RT             | F | <b>60 - 75</b> | Rectum            | <b>No</b> | No      | <b>5-Fluorouracile, oxaliplatin, cetuximab</b>        |
| P11  | Liver | High-RT           | M | 60 - 75        | Left colon        | No        | No      | 5-Fluorouracile, oxaliplatin, panitumumab             |

|       |       |         |   |                |               |                |                |                                                          |
|-------|-------|---------|---|----------------|---------------|----------------|----------------|----------------------------------------------------------|
| P11*  |       | No-RT   | M | 60 - 75        | Left colon    | No             | No             | 5-Fluorouracile, oxaliplatin,<br>panitumumab             |
| P12   | Colon | High-RT | M | 60 - 75        | Left colon    | Unknown        | Unknown        | None                                                     |
| P12*  |       | No-RT   | M | 60 - 75        | Left colon    | Unknown        | Unknown        | None                                                     |
| P13   | Colon | High-RT | M | > 75           | Left colon    | No             | No             | None                                                     |
| P13*  |       | No-RT   | M | > 75           | Left colon    | <b>Unknown</b> | <b>Unknown</b> | None                                                     |
| P14C  | Colon | High-RT | F | > 75           | Left colon    | No             | No             | 5-Fluorouracile, oxaliplatin                             |
| P14C* |       | No-RT   | F | <b>60 - 75</b> | Left colon    | No             | No             | <b>5-Fluorouracile, oxaliplatin,<br/>panitumumab</b>     |
| P14L  | Liver | High-RT | F | > 75           | Left colon    | No             | No             | 5-Fluorouracile, oxaliplatin                             |
| P14L* |       | No-RT   | F | <b>60 - 75</b> | <b>Rectum</b> | No             | No             | <b>5-Fluorouracile, oxaliplatin,<br/>cetuximab</b>       |
| P15   | Colon | High-RT | F | > 75           | Left colon    | No             | No             | 5-Fluorouracile, oxaliplatin, cetuximab                  |
| P15*  |       | No-RT   | F | <b>60 - 75</b> | Left colon    | No             | No             | <b>5-Fluorouracile, oxaliplatin,<br/>panitumumab</b>     |
| P16   | Liver | High-RT | M | 60 - 75        | Right colon   | No             | No             | 5-Fluorouracile, oxaliplatin                             |
| P16*  |       | No-RT   | M | 60 - 75        | Right colon   | No             | No             | 5-Fluorouracile, oxaliplatin                             |
| P18   | Liver | High-RT | M | 60 - 75        | Left colon    | Yes            | No             | 5-Fluorouracile, oxaliplatin,<br>irinotecan, bevacizumab |
| P18*  |       | No-RT   | M | 60 - 75        | Left colon    | Yes            | No             | 5-Fluorouracile, oxaliplatin,<br>irinotecan, bevacizumab |

*Table A10.2: Characteristics used for matching irradiated patients with never-irradiated patients to establish the No-RT lesion cohort. Bold values indicate discordant characteristics between matched patients. Patients marked with \* differ from their matched irradiated counterpart, whereas for patients without \*, a No-RT lesion resected prior to irradiation was available. F: Female, M: Male, RT: Radiotherapy.*

| Antigen retrieval | Primary antibody |          | Secondary antibody | Tyramide     |          |
|-------------------|------------------|----------|--------------------|--------------|----------|
|                   | Target           | Dilution |                    | Fluorochrome | Dilution |
| Acidic (pH 5.7)   | CD3              | 1:1000   | Anti-rabbit        | CF754        | 1:1000   |
| Acidic (pH 5.7)   | CD8              | 1:1000   | Anti-mouse         | CF647        | 1:1000   |
| Acidic (pH 5.7)   | CD45RO           | 1:200    | Anti-mouse         | AF594        | 1:200    |
| Basic (pH 9)      | PD-1             | 1:1000   | Anti-mouse         | CF488        | 1:1000   |
| Basic (pH 9)      | PD-L1            | 1:500    | Anti-rabbit        | CF555        | 1:500    |

*Table A10.3: Panel of antibodies used for multiplex immunofluorescence staining. AF: Alexa Fluor, CF: Compact Fluorophores.*

| Reagent/resource                                    | Reference               | Identifier    |
|-----------------------------------------------------|-------------------------|---------------|
| <b>FACS</b>                                         |                         |               |
| Vacutainer® CPT™ Mononuclear Cell Preparation Tubes | BD Bioscience           | # 362782      |
| Iscove's Modified Dulbecco's Medium (IMDM)          | Gibco™                  | # 12440053    |
| Human Serum (Heat inactivated)                      | Merck Life Science B.V. | # H5667-100ML |
| Dimethyl sulfoxide                                  | Sigma Aldrich           | # 506008      |
| NovoCyte Quanteon Flow Cytometer Systems 4 Lasers   | Agilent                 | NA            |
| FlowJo™ Software (v10.10.0.)                        | BD Bioscience           | NA            |
| CCR6 - APC R700                                     | BD Bioscience           | # 565173      |
| CCR7 - BV786                                        | BD Biosciences          | # 566758      |
| CD11c - PE-Cy5                                      | Biolegend               | # 337206      |
| CD123 - FITC                                        | Biolegend               | # 306014      |
| CD127 - APC                                         | Biolegend               | # 351316      |
| CD137 - RB705                                       | BD Biosciences          | # 570577      |
| CD14 - BB700                                        | BD Bioscience           | # 566465      |
| CD16 - APC R700                                     | BD Bioscience           | # 566969      |
| CD19 - APC                                          | Biolegend               | # 302212      |
| CD25 - SYG 581                                      | Biolegend               | # 356154      |
| CD27 - NFY610                                       | BD Biosciences          | # H012T02Y03  |
| CD28 - APC                                          | Biolegend               | # 377610      |
| CD3 - BV570                                         | Biolegend               | # 300436      |

|                       |                          |               |
|-----------------------|--------------------------|---------------|
| CD3 - Spark Blue 574  | Biolegend                | # 300488      |
| CD33 - BV711          | Biolegend                | # 303424      |
| CD38 - BV785          | Biolegend                | # 303530      |
| CD39 - BV421          | Biolegend                | # 328214      |
| CD4 - FITC            | Biolegend                | # 300506      |
| CD45 - APC-Cy7        | Biolegend                | # 304014      |
| CD45 - PE-Fire700     | Biolegend                | # 304078      |
| CD45RA - APC-Fire750  | Biolegend                | # 304152      |
| CD56 - BV605          | Biolegend                | # 318334      |
| CD69 - RY775          | BD Biosciences           | # 571396      |
| CD8 - BV510           | Biolegend                | # 344732      |
| CTLA-4 - BV711        | Biolegend                | # 369631      |
| CXCR3 - PE-Dazzle 594 | Biolegend                | # 353736      |
| DAPI                  | Milteny                  | # 130-111-570 |
| HLA DR - BV650        | Biolegend                | # 307650      |
| LAG-3 - NFY730        | ThermoFischer Scientific | # H048T02Y07  |
| PD-1 - RB780          | BD Biosciences           | # 568703      |
| TIGIT - NF red 700    | ThermoFischer Scientific | # H011T02R03  |
| TIM3 - SB645          | ThermoFischer Scientific | # 64310942    |
| Zombie Yellow         | Biolegend                | # 423103      |

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**Proteomics**

|                                                     |                          |          |
|-----------------------------------------------------|--------------------------|----------|
| High-Select™ Top14 Abundant Protein Depletion Resin | ThermoFischer Scientific | # A36371 |
|-----------------------------------------------------|--------------------------|----------|

|                                                                    |                          |             |
|--------------------------------------------------------------------|--------------------------|-------------|
| High-Select™ Top14 Abundant Protein Depletion<br>Midi Spin Columns | ThermoFischer Scientific | # A36372    |
| Sequencing Grade Modified Trypsin                                  | Promega                  | # V5111     |
| Pierce™ High pH Reversed-Phase Peptide<br>Fractionation Kit        | ThermoFischer Scientific | # 84868     |
| Orbitrap Exploris™ 240 Mass Spectrometer                           | ThermoFischer Scientific | # BRE725535 |

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**Multiplex immunofluorescence**


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|                                                        |                          |                         |
|--------------------------------------------------------|--------------------------|-------------------------|
| Epredia™ Lames adhésives SuperFrost Plus™              | ThermoFischer Scientific | # 10149870              |
| Bovine Serum Albumin                                   | Sigma                    | # A9418-50G             |
| Mouse monoclonal anti-CD45RO (IF)                      | Cell Signaling           | # 55618, clone UCHL1    |
| Mouse monoclonal anti-CD8 (IF)                         | Agilent                  | # M7103, clone C8/144B  |
| Mouse monoclonal anti-PD-1 (IF)                        | Abcam                    | # ab52587, clone NAT105 |
| Rabbit monoclonal anti-CD3 ε antibody (IF)             | Abcam                    | # ab16669, clone SP7    |
| Rabbit monoclonal anti-PD-L1 (IF)                      | Cell Signaling           | # 13684, clone E1L3N    |
| EnVision+ System- HRP Labelled Polymer Anti-<br>Rabbit | Agilent                  | # K4003                 |
| EnVision+ System- HRP Labelled Polymer Anti-<br>Mouse  | Agilent                  | # K4001                 |
| Tyramide-CF488                                         | Biotium                  | # 92171                 |
| Tyramide-CF555                                         | Biotium                  | # 96020                 |
| Alexa Fluor™ 594                                       | ThermoFischer Scientific | # B40957                |
| Tyramide-CF647                                         | Biotium                  | # 96022                 |
| Tyramide-CF754                                         | Biotium                  | # 96090                 |

|                                                                        |                          |            |
|------------------------------------------------------------------------|--------------------------|------------|
| Hoechst 33342, trihydrochloride trihydrate, 10 mg/mL solution in water | ThermoFischer Scientific | # H3570    |
| Fluorescence mounting medium                                           | DAKO                     | # S3023    |
| Borosilicate Cover Glass                                               | VWR                      | # 631-0146 |
| Axioscan Z1                                                            | Zeiss                    | N/A        |
| <b>RNA-seq</b>                                                         |                          |            |
| RNeasy FFPE Kit for RNA Extraction                                     | Qiagen                   | # 73504    |
| Deparaffinization Solution                                             | Qiagen                   | # 19093    |
| NanoDrop 1000 Spectrophotometer                                        | ThermoFischer Scientific | N/A        |
| 2200 TapeStation System                                                | Agilent                  | N/A        |
| Watchmaker RNA Polaris Depletion - rRNA/Globin (FFPE)                  | Watchmaker Genomics      | N/A        |
| NovaSeq X Plus                                                         | Illumina                 | N/A        |

*Table A10.4: References of the resources used for the biomolecular analyses. FACS: Fluorescence-activated cell sorting.*

### **7.10.1. Detailed results on feasibility and safety**

#### **7.10.1.1. Radiotherapy**

Among the 18 included patients, 11 received 3 x 8 Gy to a liver metastasis, 3 to a lung metastasis, 3 to a primary tumour, and 1 patient received treatment to both a liver metastasis and a primary tumour. Three patients received 3 x 1 – 2 Gy to a second liver metastasis (“Low-RT”), and in six patients, a metastasis (five liver, one lung) was not irradiated (“Out-RT”). One patient treated for a liver metastasis also received concomitant short-course pelvic RT (5 x 5 Gy). In another patient with multiple liver metastases, only the “Mod-RT” lesion was resected; “Low-RT” and “Out-RT” lesions were not removed during the surgical procedure. The median GTV volume for High-RT lesions was 9.6 cm<sup>3</sup> (interquartile range: 5.7 – 31.0cm<sup>3</sup>) and 4.6cm<sup>3</sup> (interquartile range: 1.5 – 12.8cm<sup>3</sup>). GTVs for Low-RT lesions were 7.1, 25.0, and 4.0cm<sup>3</sup>. Of the 100 evaluated target volume coverage objectives, 84.0% were met (Table A10.5). All organs-at-risk constraints were respected except for one patient with a liver metastasis adjacent to the stomach and duodenum. In this case, dose limits were slightly exceeded: stomach D0.1cc = 22.8Gy (limit: 22.2Gy), duodenum D0.1cc = 26.9 Gy (limit: 25.2Gy), and duodenum D10cc = 21.0 Gy (limit: 11.4Gy). The patient was closely monitored for gastrointestinal toxicity. As prophylaxis, oral ondansetron (8mg) was administered 30 minutes before each session to prevent vomiting, and pantoprazole (40mg) was prescribed for 2 months to prevent gastro-duodenal ulceration. Regarding RT-related adverse events, four patients experienced Grade 1 fatigue. One patient developed Grade 2 vomiting, beginning on the first day of RT and persisting until the day before



surgery. Due to its onset just before RT sessions and resistance to treatment, the symptom can be considered more likely anticipatory than RT-induced. Notably, stomach and duodenum doses in this patient were well below organ-at-risk constraints. All RT treatments were completed within three consecutive days.

| Volume  | Objective        | Median value<br>(interquartile range,<br>Gy) | Respected objectives /<br>N evaluated lesions |
|---------|------------------|----------------------------------------------|-----------------------------------------------|
| GTV_Mod | D0,1cc < 2880cGy | 26.2 (25.6 – 27.4)                           | 18/19                                         |
|         | D98% > 2400cGy   | 24.4 (24.1 – 25.6)                           | 15/19                                         |
| GTV_Low | D0,1cc < 600cGy  | 4.3, 5.2, and 5.5                            | 3/3                                           |
|         | D100% > 300cGy   | 3.8, 4.3 and 4.5                             | 3/3                                           |
| GTV_Out | Dmean < 50cGy    | 0.3 (0.3 – 0.6)                              | 4/6                                           |
| PTV_Mod | D0,1cc < 2880cGy | 26.2 (25.8 – 27.6)                           | 18/19                                         |
|         | D95% > 2400cGy   | 24.0 (23.9 – 24.2)                           | 15/19                                         |
| PTV_Low | D0,1cc < 600cGy  | 4.8, 6.1, and 7.1                            | 1/3                                           |
|         | D100% > 300cGy   | 3.1, 3.1, and 4.2                            | 3/3                                           |
| PTV_Out | Dmean < 50cGy    | 0.3 (0.3 – 0.6)                              | 4/6                                           |

*Table A10.5: Target volume coverage results. GTV: Gross tumour volume, PTV: Planning target volume.*

#### 7.10.1.2. Surgery

The median interval between RT and surgery was 5.0 days (interquartile range: 5.0 – 6.0), with no delays in planned surgeries due to the RT protocol. Median surgical duration and total in-room time were 282 minutes (interquartile range: 217 – 358) and 398 minutes (interquartile range: 328 – 458), respectively. Median intraoperative blood loss was 250 mL (range: minimal to 438 mL). Complete resection was achieved in 23 of 26 lesions. In patient 17, preoperative imaging and clinical history suggested a lung

metastasis, but pathology revealed a benign lesion consistent with necrotizing granulomatous pneumonitis.

One intraoperative complication occurred: a case of aeric leakage following lung metastasectomy. Median postoperative hospital stay was 6.0 days (interquartile range: 5.0 – 7.0). No postoperative complications were attributed to preoperative RT (Table A10.3). All complications resolved with appropriate management, and no patient required reoperation or early readmission.

Four of the six participating surgeons completed the feasibility survey (Figure A10.1). While all initially expressed concerns regarding the potential impact of RT on surgical outcomes, these were alleviated after performing at least one case. Only one surgeon noted increased fibrosis in the irradiated field, but no significant effects were reported on blood loss, operative time, or postoperative complications.

| Patient | Surgery                                     | Postoperative complications                           | Number of resected lesions | RT-group | Lesion size (mm) | Resection status | Tumour regression grade |
|---------|---------------------------------------------|-------------------------------------------------------|----------------------------|----------|------------------|------------------|-------------------------|
| 1       | Extended right hepatectomy                  | Vesical globus, <i>Klebsiella Oxycata</i> bacteraemia | 11                         | High-RT  | 22               | R0               | 4                       |
|         |                                             |                                                       |                            | Low-RT   | 20               | R0               | 4                       |
|         |                                             |                                                       |                            | Out-RT   | 22               | R0               | 4                       |
| 2       | Left hepatectomy                            | Transient hepatic encephalopathy (small-for-size)     | 3                          | High-RT  | 65               | R0               | 4                       |
|         |                                             |                                                       |                            | Out-RT   | 39               | R1               | 4                       |
| 3       | Left lobectomy (liver)                      | -                                                     | 2                          | High-RT  | 15               | R0               | 1                       |
|         |                                             |                                                       |                            | Low-RT   | 130              | R1               | 2                       |
|         |                                             |                                                       |                            | Out-RT   | 130              | R1               | 2                       |
| 4       | Bisegmentectomy and wedge resection (liver) | Pyrexia, Serous collection drainage                   | 2                          | High-RT  | 60               | R0               | 3                       |
|         |                                             |                                                       |                            | Out-RT   |                  | R0               | 2                       |
| 5       | Right hepatectomy                           | -                                                     | 6                          | High-RT  | 65               | R0               | 1                       |
| 6       | Wedge resection (lung)                      | -                                                     | 1                          | High-RT  | 7                | R0               | -                       |
| 7       | Segmentectomy (liver)                       | -                                                     | 2                          | High-RT  | 27               | R0               | 4                       |
| 8       | Wedge resection (liver)                     | -                                                     | 1                          | High-RT  | 32               | R0               | 3                       |
| 9       | Wedge resection (lung)                      | Pneumothorax                                          | 3                          | High-RT  | 10               | R0               | -                       |
|         |                                             |                                                       |                            | Out-RT   |                  | R0               | -                       |
| 10      | Segmentectomy (liver)                       | Ileostomy stenosis                                    | 1                          | High-RT  | 27               | R0               | 4                       |
| 11      | Left hepatectomy                            | -                                                     | 1                          | High-RT  | 78               | R0               | 3                       |

|    |                            |   |   |         |    |           |   |
|----|----------------------------|---|---|---------|----|-----------|---|
| 12 | Left hemicolectomy         | - | 1 | High-RT | 41 | R0        | 5 |
| 13 | Sigmoidectomy              | - | 1 | High-RT | 17 | R0        | 5 |
| 14 | Left hemicolectomy         |   | 1 | High-RT | 70 | R0        | 5 |
|    | Wedge resection<br>(liver) | - | 1 | High-RT | 6  | R0        | 2 |
| 15 | Left hemicolectomy         | - | 1 | High-RT | 15 | R0        | 5 |
| 16 | Wedge resection<br>(liver) | - | 1 | High-RT | 25 | R0        | 3 |
| 17 | Wedge resection<br>(lung)  | - | 1 | High-RT |    | No tumour |   |
| 18 | Left lobectomy (liver)     | - | 1 | High-RT | 45 | R0        | 3 |

Table A10.6: Surgery characteristics and results. RT: Radiotherapy;

Before the study start, I had a negative preconception about the RT impact on the surgery

I noticed increased fibrosis compared to a non-irradiated patient

I noticed greater blood loss compared to a non-irradiated patient

The duration of the procedure seems longer compared to a non-irradiated patient

My resection is wider/does not allow to preserve as much healthy tissue compared to a non-irradiated patient

Postoperative hospitalisation seems more complicated compared to a non-irradiated patient

Overall, I noticed a difference compared to a non-irradiated patient

Today, I feel confident when operating an irradiated patient

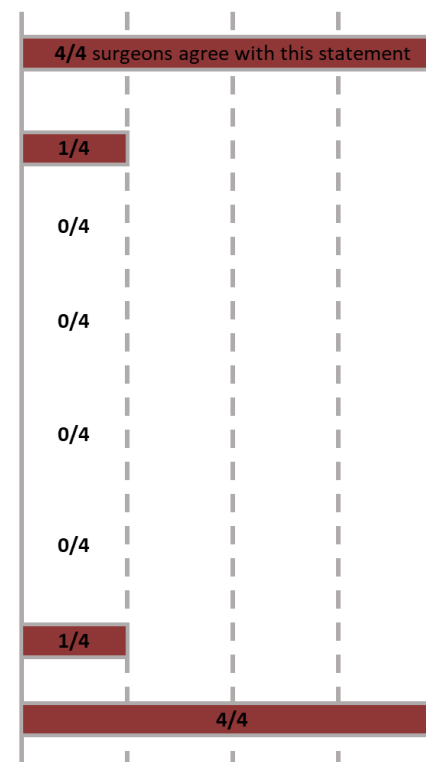


Figure A10.1: Results of the surgeon survey. RT: Radiotherapy.

#### **7.10.1.3. Follow-up**

The median follow-up was 9.0 months (interquartile range: 5.0 – 12.8). The 30-day morbidity rate, assessable in 16 of 18 patients, was 12.5%. One patient experienced persistent postoperative pain requiring oral analgesics (paracetamol and tramadol), and another developed severe malnutrition. The 90-day mortality rate, also assessed in 16 patients, was 0%.

Local relapse following liver metastasis resection occurred in three patients at 10.2, 10.4, and 16.4 months post-surgery. Distant progression was observed in five patients, all initially treated for liver metastases, including: portal lymph node recurrence (3.2 months), lung and brain metastases (4.4 months), isolated lung metastasis (7.0 months), peritoneal carcinomatosis (7.7 months), and lung metastasis (8.4 months). At the latest follow-up, all patients were alive.

#### **7.10.2. Detailed results of the FACS analysis**

The immune population panel enabled to determined subsets of immune cells among the general populations and to compare it between the blood Pre-RT and the blood Post-RT

- CD8<sup>+</sup> T cells : central memory (CM), effector memory (EM), terminally-differentiated effector memory RA<sup>+</sup> (TEMRA), and naïve: no statistical difference ( $p > 0.05$ ).
- CD4<sup>+</sup> T cells : central memory (CM), effector memory (EM), terminally-differentiated effector memory RA<sup>+</sup> (TEMRA), and naïve:

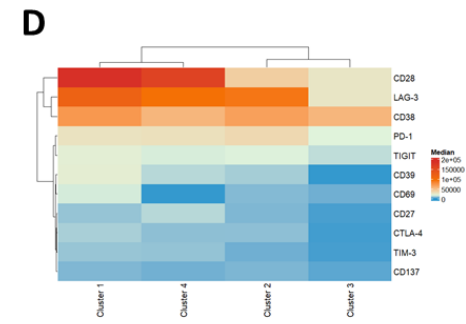
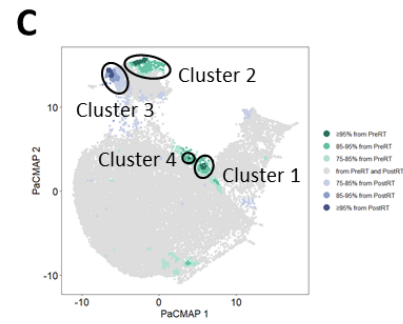
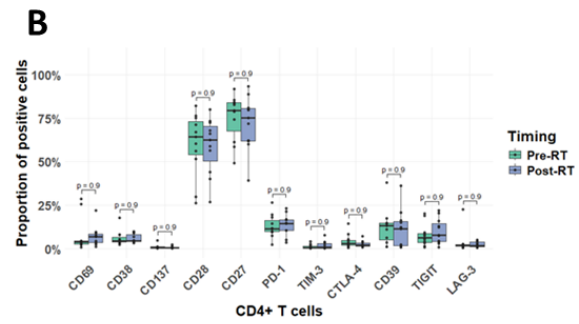
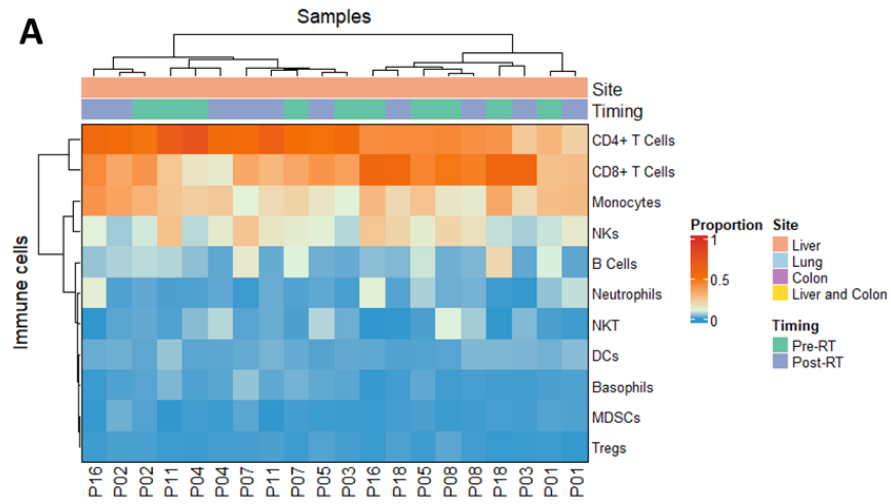
a significant higher proportion of terminally-differentiated effector memory RA<sup>+</sup> (TEMRA) CD4<sup>+</sup> T cells was found in the blood of patients before irradiation ( $p = 0.049$ )

- CD4<sup>+</sup> T cells : Th1, Th2, and Th17: no statistical difference ( $p > 0.05$ ).
- Monocytes : classical, intermediate, non-classical: no statistical difference ( $p > 0.05$ ).
- Myeloid-derived suppressor cells : granulocytic-myeloid-derived suppressor cells and monocytic-myeloid-derived suppressor cells: no statistical difference ( $p > 0.05$ ).
- dendritic cells : pndendritic cells and Modendritic cells: no statistical difference ( $p > 0.05$ ).
- Natural killers : early, mature, and terminal: no statistical difference ( $p > 0.05$ ).

Analysis of the activation/exhaustion state of CD4<sup>+</sup> and CD8<sup>+</sup> cells in patients irradiated on liver metastases only demonstrated not significant difference regarding each marker individually ( $p > 0.05$  for both CD4<sup>+</sup> and CD8<sup>+</sup> cells, Figure A10.2B and Figure A10.2E). When using T-REX analysis, 4 clusters of CD4<sup>+</sup> T cells were highlighted: clusters 1, 4 (similar to cluster 4 of the whole population) and 2 representing cells from Pre-RT blood, while cluster 3 encompass cells from the Post-RT blood, which is similar to cluster 3 of the whole population (Figure A10.2C). Compared to Pre-RT clusters, post-RT blood are enriched in CD4<sup>+</sup> T cells that expressed lower marker of naivity (CD27 and CD28) as well as lower inhibition markers (PD-1, TIM-3, CTLA-4, CD39, and LAG-3, Figure A10.2D). For CD8<sup>+</sup> cells, 2 clusters of cells, both from

Pre-RT blood were identified (Figure A10.2F). Compared to other cells they are characterised by a more inhibited pattern (higher expression of PD-1, TIM-3, CTLA-4, CD39, and LAG-3,  $p < 0.001$ , Dunn test), a loss of naivety (lower expression of CD27 and CD28,  $p < 0.001$ , Dunn test) and a lower activation level (lower expression of CD38 and CD69,  $p < 0.001$ , Dunn test, Figure A10.2G).





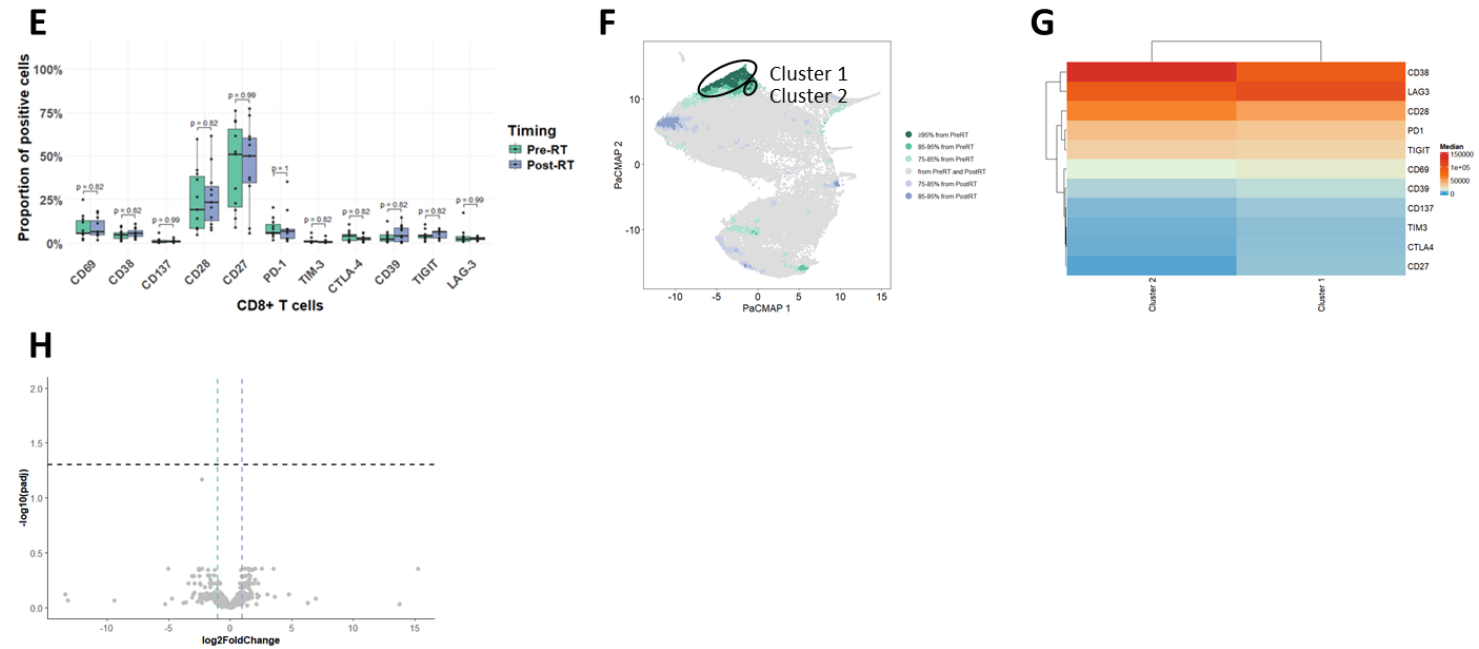


Figure A10.2: Blood results for patients irradiated on liver metastases only ( $n = 10$ ). (A) Proportions of circulating immune cell populations according to RT timing (Pre-RT versus Post-RT) and irradiation site. (B) Single expression of activation/exhaustion markers in  $CD4^+$  T cells comparing Pre-RT and Post-RT blood. (C) Dimensionality reduction with unsupervised clustering of  $CD4^+$  T cells based on activation/exhaustion marker expression identified four clusters. Clusters 1, 2, and 4 were enriched in Pre-RT blood, while cluster 3 was enriched in Post-RT blood. (D) Expression of activation/exhaustion markers within each  $CD4^+$  T-cell cluster. (E) Single expression of

activation/exhaustion markers in CD8<sup>+</sup> T cells comparing Pre-RT and Post-RT blood. (F) Dimensionality reduction with unsupervised clustering of CD8<sup>+</sup> T cells based on activation/exhaustion marker expression identified two clusters, both comprising cells from pre-RT blood. (G) Expression of activation/exhaustion markers within each CD8<sup>+</sup> T-cell cluster. (H) Differential analysis of plasmatic proteins showed no significant differences between Pre-RT and Post-RT blood.

### 7.10.3. Detailed results of the proteomic analysis

Proteins associated with activation of the p53 pathway were differentially expressed in Post-RT compared with Pre-RT blood samples, suggesting that RT may trigger p53-related signalling within tumours, leading to the systemic release of these proteins. Among the detected proteins (n = 29), several are known tumour suppressors: levels of AK1, BAX, HINT1, SERPINB5, STOM, and VAMP8 decreased after RT, whereas CD82, CDH13, CTSF, CYFIP2, FUCA1, ST14, STEAP3, and VWA5A increased. Conversely, some proteins have recognised pro-oncogenic roles: APP and TGfree-breathing1 decreased following RT, while DNTTIP2, GM2A, PLXNB2, S100A4, SEC61A1, SLC3A2, and TCN2 increased (Table A10.7).

|                 |               |                  |                |                |
|-----------------|---------------|------------------|----------------|----------------|
| ABAT            | ABCC5         | ABHD4            | ACVR1B         | <b>ADA</b> ↗   |
| AEN             | <b>AK1</b> ↘  | ALOX15B          | ANKRA2         | APAF1          |
| <b>APP</b> ↘    | ATF3          | BAIAP2           | BAK1           | <b>BAX</b> ↘   |
| BLCAP           | BMP2          | BTG1             | BTG2           | CASP1          |
| CCND2           | CCND3         | CCNG1            | CCNK           | CCP110         |
| CD81            | <b>CD82</b> ↗ | <b>CDH13</b> ↗   | CDK5R1         | CDKN1A         |
| CDKN2A          | CDKN2AIP      | CDKN2B           | CEBPA          | CGRRF1         |
| CLCA2           | COQ8A         | CSRNP2           | CTSD           | <b>CTSF</b> ↗  |
| <b>CYFIP2</b> ↗ | DCXR          | DDB2             | DDIT3          | DDIT4          |
| DEF6            | DGKA          | <b>DNTTIP2</b> ↗ | DRAM1          | EI24           |
| ELP1            | EPHA2         | EPHX1            | EPS8L2         | ERCC5          |
| F2R             | FAM162A       | FAS              | FBXW7          | FDXR           |
| FGF13           | FOS           | FOXO3            | <b>FUCA1</b> ↗ | GADD45A        |
| GLS2            | <b>GM2A</b> ↗ | GPX2             | H1-2           | H2AC25         |
| H2AJ            | HBEGF         | HDAC3            | HEXIM1         | <b>HINT1</b> ↘ |
| HMOX1           | HRAS          | HSPA4L           | IER3           | IER5           |
| IFI30           | IL1A          | INHBB            | IP6K2          | IRAG2          |
| IRAK1           | ISCU          | ITGB4            | JAG2           | JUN            |
| KIF13B          | KLF4          | KLK8             | KRT17          | <b>LDHB</b> ↗  |
| LIF             | MAPKAPK3      | MDM2             | MKNK2          | MXD1           |

|                 |               |                            |                   |                 |
|-----------------|---------------|----------------------------|-------------------|-----------------|
| MXD4            | NDRG1         | NHLH2                      | NINJ1             | NOL8            |
| NOTCH1          | NUDT15        | NUPR1                      | OSGIN1            | PCNA            |
| PDGFA           | PERP          | PHLDA3                     | PIDD1             | PITPNC1         |
| PLK2            | PLK3          | <b>PLXNB2</b> ↗            | PMM1              | POLH            |
| POM121          | PPM1D         | PPP1R15A                   | PRKAB1            | PRMT2           |
| <b>PROCR</b> ↗  | PTPN14        | PTPRE                      | PVT1              | RAB40C          |
| <b>RACK1</b> ↗  | RAD51C        | RAD9A                      | RALGDS            | <b>RAP2B</b> ↗  |
| RB1             | RCHY1         | RETSAT                     | RGS16             | RHBDF2          |
| RNF19B          | RPL18         | RPL36                      | <b>RPS12</b> ↗    | RPS27L          |
| RRAD            | RRP8          | RXRA                       | S100A10           | <b>S100A4</b> ↗ |
| SAT1            | SDC1          | <b>SEC61A1</b> ↗           | <b>SERPINB5</b> ↘ | SERTAD3         |
| SESN1           | SFN           | SLC19A2                    | SLC35D1           | <b>SLC3A2</b> ↗ |
| SLC7A11         | SOCS1         | SP1                        | SPHK1             | <b>ST14</b> ↗   |
| <b>STEAP3</b> ↗ | <b>STOM</b> ↘ | TAP1                       | TAX1BP3           | TCHH            |
| <b>TCN2</b> ↗   | TGFA          | <b>TGfree-breathing1</b> ↘ | TM4SF1            | TM7SF3          |
| TNFSF9          | TNNI1         | TOB1                       | TP53              | TP63            |
| TPD52L1         | TPRKB         | TRAF4                      | TRAFD1            | TRIAP1          |
| TRIB3           | TSC22D1       | TSPYL2                     | TXNIP             | UPP1            |
| <b>VAMP8</b> ↘  | VDR           | <b>VWA5A</b> ↗             | WRAP73            | WWP1            |
| XPC             | ZBTB16        | ZFP36L1                    | ZMAT3             | ZNF365          |

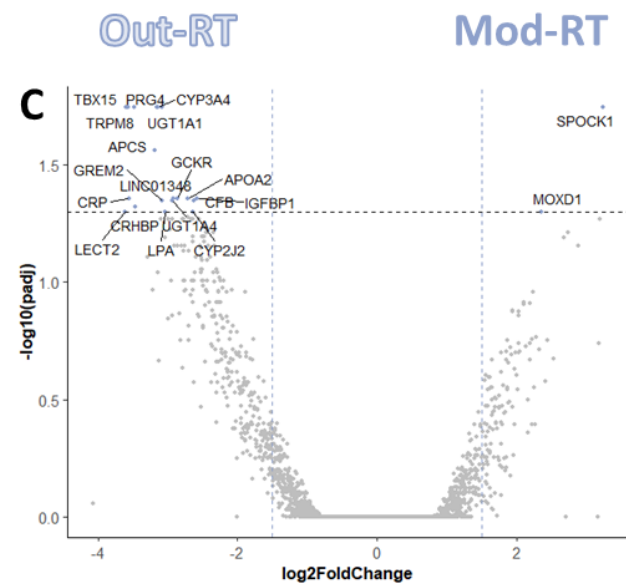
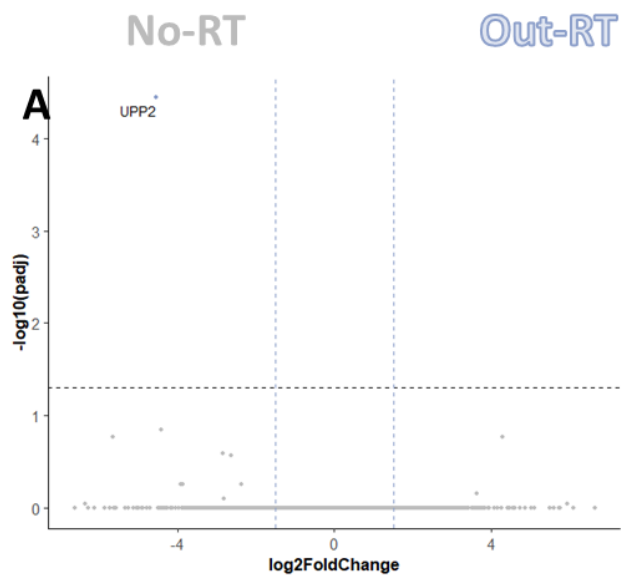
Table A10.7: Genes included in the p53 hallmark pathway, which were significantly overexpressed in post-RT versus pre-RT blood samples. Among these, 29 genes (14.5%) were detected in at least 70% of samples (bold). Of these, 21 were upregulated (↗) and 8 were downregulated (↘).

#### 7.10.4. Detailed results of the RNA-sequencing

Only *UPP2*, a gene involved in the uridine catabolic process, was significantly overexpressed in No-RT compared to the two Out-RT samples (Figure A10.3A). Out-RT samples exhibited significant enrichment of two metabolic pathways as well as the TNF- $\alpha$  (via NF- $\kappa$ B) signalling pathway. Acute activation of this pathway, through pro-inflammatory mediators (cytokines, chemokines, inflammasome), is linked to Th1/Th17 CD4<sup>+</sup> T-cell

differentiation, activation and maturation into memory cells, M1 macrophage polarization, dendritic cell maturation, and neutrophil recruitment, but also to Treg proliferation (Figure A10.3B) [578].

Comparison of Out-RT and Mod-RT samples showed fewer proliferation-related genes and pathways overexpressed in Out-RT than in the No-RT versus Mod-RT comparison, suggesting less proliferative tumours compared to Mod-RT. Conversely, metabolic genes and pathways were significantly enriched in Out-RT, consistent with preserved liver metabolic function compared to Mod-RT. Finally, several immune-related pathways (IFN- $\alpha$ , IFN- $\gamma$ , inflammatory response, and allograft rejection) were overexpressed in Mod-RT, indicating a more pro-inflammatory tumour immune microenvironment in tumours irradiated with 3 x 8 Gy (Figure A10.3C-D).



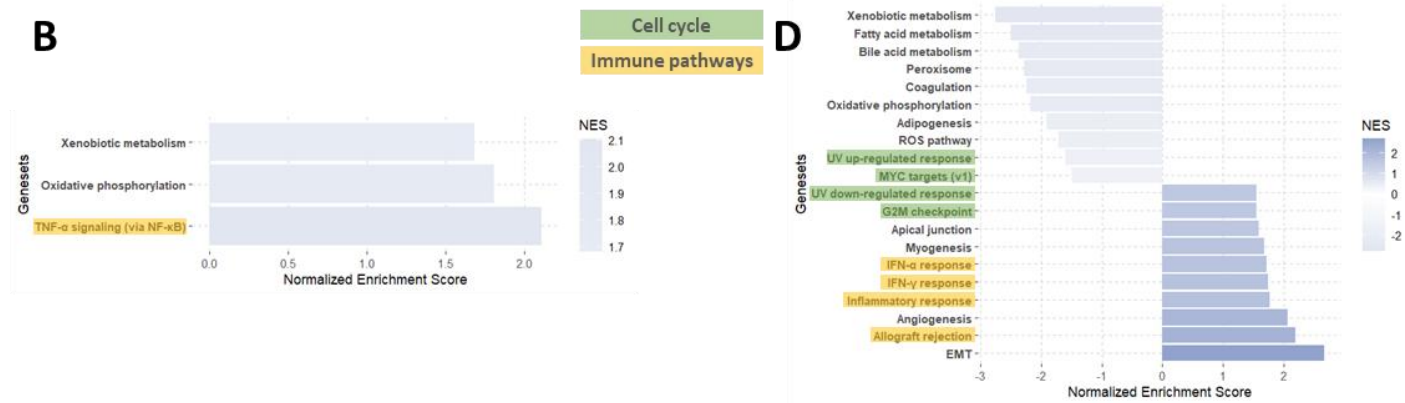


Figure A10.3: Differential gene expression (A) and cancer hallmark pathway enrichment (B) between No-RT and Out-RT samples ( $n = 2$ ). Same analyses were performed between Out-RT and Mod-RT samples.



| No-RT versus Mod-RT   |                                                                 |                                                                        |                       |                                              |                                                                 |
|-----------------------|-----------------------------------------------------------------|------------------------------------------------------------------------|-----------------------|----------------------------------------------|-----------------------------------------------------------------|
| No-RT                 |                                                                 |                                                                        | Mod-RT                |                                              |                                                                 |
| Gene                  | Gene name                                                       | Gene function                                                          | Gene                  | Gene name                                    | Gene function                                                   |
| <i>AGR2</i>           | Anterior Gradient 2, Protein Disulphide Isomerase Family Member | Cell migration, cellular transformation, metastasis, and p53 inhibitor | <i>CDKN1A</i>         | Cyclin dependent kinase inhibitor 1A (= p21) | Tumour suppressor                                               |
| <i>AGR3</i>           | Anterior Gradient 3, Protein Disulphide Isomerase Family Member | Ciliary beat frequency in the airway                                   | <i>DNAI3</i>          | Dynein Axonemal Intermediate Chain 3         | Negative regulation of cell migration, invasion, and metastasis |
| <i>BLACA T1</i>       | BLACAT1 Overlapping LEMD1 Locus                                 | Cell proliferation at G0/G1 checkpoint                                 | <i>EDN2</i>           | Endothelin 2                                 | Vasoconstriction                                                |
| <i>CAPN8</i>          | Calpain 8                                                       | Proteolysis                                                            | <i>ENSG0000289914</i> | -                                            | Long non-coding RNA                                             |
| <i>CDC20</i>          | Cell Division Cycle 20                                          | Anaphasis promotion                                                    | <i>ENSG0000302015</i> | -                                            | Long non-coding RNA                                             |
| <i>CDX1</i>           | Caudal Type Homeobox 1                                          | Intestine-specific gene regulation and enterocyte differentiation      | <i>IGSF21</i>         | Immunoglobulin Superfamily Member 21         | Immunoglobulin                                                  |
| <i>CLDN4</i>          | Claudin 4                                                       | Epithelial cell tight junction component                               | <i>MIR34AHG</i>       | MIR34A Host Gene                             | Long non-coding RNA                                             |
| <i>ENSG0000272141</i> | -                                                               | Long non-coding RNA                                                    | <i>MIR3659HG</i>      | MIR3659 Host Gene                            | Long non-coding RNA                                             |

|                       |                                                     |                                  |                     |                                                                            |                                            |
|-----------------------|-----------------------------------------------------|----------------------------------|---------------------|----------------------------------------------------------------------------|--------------------------------------------|
| <i>ENSG0000295244</i> | -                                                   | Long non-coding RNA              | <i>PTCHD4</i>       | Patched Domain Containing 4                                                | Hedgehog inhibition                        |
| <i>ENSG0000298364</i> | -                                                   | Long non-coding RNA              | <i>SPATA18</i>      | Spermatogenesis Associated 18                                              | Mitochondrial quality control              |
| <i>ENSG0000309156</i> | -                                                   | Long non-coding RNA              | <i>THBS2-AS1</i>    | THBS2 Antisense RNA 1                                                      | Long non-coding RNA                        |
| <i>EPCAM</i>          | Epithelial Cell Adhesion Molecule                   | Cell adhesion                    | <i>ZMAT3</i>        | Zinc Finger Matrin-Type 3                                                  | Cell growth inhibition                     |
| <i>GNG4</i>           | G Protein Subunit Gamma 4                           | Cell proliferation and migration | <i>LINC03040</i>    | Long Intergenic Non-Protein Coding RNA 3040                                | Long non-coding RNA                        |
| <i>GPA33</i>          | Glycoprotein A33                                    | Plasma membrane immunoglobulin   | <i>LOC100421651</i> | Caseinolytic Mitochondrial Matrix Peptidase Chaperone Subunit X Pseudogene | Pseudogene                                 |
| <i>HHLA2</i>          | HHLA2 Member of B7 Family                           | T cell proliferation inhibition  | <i>LRATD1</i>       | LRAT Domain Containing 1                                                   | Cell morphogenesis and motility            |
| <i>IGF2BP3</i>        | Insulin Like Growth Factor 2 mRNA Binding Protein 3 | RNA synthesis and metabolism     | <i>MEP1A</i>        | Meprin A Subunit Alpha                                                     | Metalloprotease                            |
| <i>IHH</i>            | Indian Hedgehog Signaling Molecule                  | Embryonic morphogenesis          | <i>MST1R</i>        | Macrophage Stimulating 1 Receptor (=RON)                                   | Inflammation attenuation in healthy tissue |
| <i>INAVA</i>          | Innate Immunity Activator                           | Innate immunity activation       | <i>RRM2</i>         | Ribonucleotide Reductase Regulatory Subunit M2                             | DNA synthesis                              |
| <i>IQGAP3</i>         | IQ Motif Containing GTPase Activating Protein 3     | Actin organisation               | <i>SLC9A2</i>       | Solute Carrier Family 9 Member A2                                          | Sodium-ion transport                       |

| No-RT versus Out-RT |                         |                                                     |        |           |               |
|---------------------|-------------------------|-----------------------------------------------------|--------|-----------|---------------|
| No-RT               |                         |                                                     | Out-RT |           |               |
| Gene                | Gene name               | Gene function                                       | Gene   | Gene name | Gene function |
| <i>UPP2</i>         | Uridine Phosphorylase 2 | Desoxycytidine monophosphate and uridine catabolism |        |           |               |

| Out-RT versus Mod-RT |                                                 |                                         |                  |                                                                 |                                   |
|----------------------|-------------------------------------------------|-----------------------------------------|------------------|-----------------------------------------------------------------|-----------------------------------|
| Out-RT               |                                                 |                                         | Mod-RT           |                                                                 |                                   |
| Gene                 | Gene name                                       | Gene function                           | Gene             | Gene name                                                       | Gene function                     |
| <i>APCS</i>          | APC Regulator Of Wnt Signaling Pathway          | Tumour suppressor                       | <i>MOXD1</i>     | Monooxygenase DBH Like 1                                        | Dopamine catabolic process        |
| <i>APOA2</i>         | Apolipoprotein A2                               | HDL component                           | <i>SPOCK1</i>    | SPARC (osteonectin), cwcw and kazal like domains proteoglycan 1 | Extracellular matrix glycoprotein |
| <i>CFB</i>           | Complement Factor B                             | Complement activation                   | <i>LECT2</i>     | Leukocyte Cell Derived Chemotaxin 2                             | Neutrophil chemotactism           |
| <i>CRHBP</i>         | Corticotropin Releasing Hormone Binding Protein | CRH and pituitary-adrenal inhibition    | <i>LINC01348</i> | Long Intergenic Non-Protein Coding RNA 1348                     | Long non-coding RNA               |
| <i>CRP</i>           | C-Reactive Protein                              | Complement activation and amplification | <i>LPA</i>       | Lipoprotein(A)                                                  | Cholesterol particle              |
| <i>CYP2J2</i>        | Cytochrome P450 Family 2 Subfamily J Member 2   | Catabolic monooxygenase                 | <i>PRG4</i>      | Proteoglycan 4                                                  | Proteoglycan                      |

|                       |                                               |                               |               |                                                                  |                                                                                                     |
|-----------------------|-----------------------------------------------|-------------------------------|---------------|------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| <i>CYP3A4</i>         | Cytochrome P450 Family 3 Subfamily A Member 4 | Catabolic monooxygenase       | <i>TBX15</i>  | T-Box Transcription Factor 15                                    | Embryogenic development, cellular proliferation, apoptosis resistance, and malignant transformation |
| <i>ENSG0000294496</i> | -                                             | Long non-coding RNA           | <i>TRPM8</i>  | Transient Receptor Potential Cation Channel Subfamily M Member 8 | Thermoception calcium channel                                                                       |
| <i>GCKR</i>           | Glucokinase Regulator                         | Glucokinase inhibition        | <i>UGT1A1</i> | UDP Glucuronosyltransferase Family 1 Member A1                   | Glucuronidation                                                                                     |
| <i>GREM2</i>          | Gremlin 2                                     | Organogenesis                 | <i>UGT1A4</i> | UDP Glucuronosyltransferase Family 1 Member A4                   | Glucuronidation                                                                                     |
| <i>IGFBP1</i>         | Insulin Like Growth Factor Binding Protein 1  | Cell migration and metabolism |               |                                                                  |                                                                                                     |

*Table A10.8: Differentially expressed genes using pairwise comparison between Mod-RT (n = 4), Out-RT (n = 2), and No-RT (n = 4) tumours. CRH: Corticotropin releasing hormone, DNA: Deoxyribonucleic acid, HDL: High density lipoprotein, RNA: Ribonucleic acid.*



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