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Prognostic and Predictive Factors for Colorectal Cancer

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Prognostic and Predictive Factors for Colorectal Cancer

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By

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To my parents,
sisters and their children

ABSTRACT

Colorectal cancer is the third most common cancer worldwide of which approximately 30% of cases are localized in the rectum. Rectal cancer accounts for around 700, 000 cases and 310, 000 deaths annually across the world with a global distribution that varies due to different lifestyles. Treatment of rectal cancer has evolved significantly during the past few decades, from being treated with surgery only to a multidisciplinary multimodal complex treatment plan with radiotherapy and chemotherapy. Consequently, a major improvement in oncologic outcomes has been witnessed with dramatically reduced local recurrence (LR) rates from more than 30%-40% 40 years ago to today's level of 5%-6%.

Study I aimed to find natural products in the NCI (National Cancer Institute, US) database with selective antitumoural effects towards cancer cells with a mutated p53 gene. For this purpose, we performed a screen of the NCI bank of natural extracts for substances with potential selective effects on cancer cells harbouring a mutated p53 gene. Only one of several selected natural extracts, N37063 demonstrated this selectivity towards p53 in our in vitro assay in several cancer cell lines. Two substances were purified from the extract which harboured most of the preferential cytotoxic effect in p53-mutated cancer cells.

Study II aimed to examine the spectrum of tumours in Swedish Lynch syndrome families. Lynch syndrome is characterized by hereditary colorectal cancer (CRC) that emerges at a young age. These patients experience colorectal cancer and endometrial cancer in their 40s and are predisposed to other malignant diseases that affect these individuals more than the general population. In this study, we demonstrated that urothelial cell cancer is the most common malignancy in the Swedish Lynch population after CRC and endometrial cancer. Furthermore, an increased proportion of gastric cancer, small bowel cancer, non-melanoma skin cancer, and ovarian cancer were observed.

Study III aimed to identify clinical parameters which could potentially predict a pathologic complete response (pCR) in preoperatively treated rectal cancers. Clinical parameters consisted of baseline imaging parameters of the rectal tumour and baseline pre-treatment clinical and laboratory parameters before the start of oncologic treatment. We identified associations between pCR and preoperative treatment, low carcinoembryonic antigen (CEA), non-elevated leucocytes, cT (magnetic resonance imaging [MRI]-defined T-stage), and MRI-estimated tumour length. A predictive model based on significant parameters was proposed.

Study IV aimed to examine the value of the neoadjuvant rectal score (NAR score) as a short-term surrogate endpoint for oncological outcomes, including time to recurrence (TTR), cancer-specific survival (CSS), and overall survival (OS), in rectal cancer patients treated preoperatively with short course radiotherapy (scRT), chemoradiotherapy (CRT), or one of these schedules in combination with systemic chemotherapy (scRT/CRT+CTX). We investigated correlations between NAR score for outcomes for all patients and in different treatment cohorts. A statistically significant correlation between NAR score and TTR, CSS, and OS outcomes in a treatment-dependent manner was observed. The prognostic value of NAR could be improved by combining pathological extramural vascular invasion and perineural invasion.

LIST OF SCIENTIFIC STUDIES

I. **Karimi M**, Conserva F, Mahmoudi S, Bergman J, Wiman K. G, Bykov V. J. N. Extract from Asteraceae *Brachylaena ramiflora* induces apoptosis preferentially in mutant p53-expressing human tumor cells. *Carcinogenesis* vol. 31 no. 6 pp. 1045–1053, 2010. doi:10. 1093/carcin/bgq084

II. **Karimi M**, von Salomé J, Aravidis C, Silander G, Stenmark Askmalm M, Henriksson I, Gebre-Medhin S, Frödin J-E, Björck E, Lagerstedt-Robinson K, Lindblom A, Tham E. A retrospective study of extracolonic, non-endometrial cancer in Swedish Lynch syndrome families. *Hereditary Cancer in Clinical Practice* (2018) 16:16. doi. org/10. 1186/s13053-018-0098-9

III. **Karimi M**, Osterlund P, Hammarström K, Imam I, Frödin J-E, Glimelius B. Associations between response to commonly used neo-adjuvant schedules in rectal cancer and routinely collected clinical and imaging parameters. *Cancers 2022 Dec 18;14(24):6238. doi: 10. 3390/cancers14246238*

IV. **Karimi M**, Osterlund P, Imam I, Hammarström K, Glimelius B, Frödin J-E. The strength of associations with survival of the neoadjuvant rectal score (NAR) after different preoperative treatments for rectal cancer. *(Manuscript in preparation)*

LIST OF ABBREVIATIONS

APR	Abdominoperineal resection
BRAF	v-raf murine sarcoma viral oncogene homolog B1
cCR	Clinical complete response
CEA	Carcinoembryonic antigen
CNS	Central nervous system
CRC	Colorectal cancer
CRT	Concurrent chemoradiotherapy
CSS	Cancer-specific survival
CT	Computed tomography
CTX	Chemotherapy
DFS	Disease-free survival
EGFR	Epidermal growth factor receptor
ELAPE	Extralevator APR
EMVI	Extramural vascular invasion
ERUS	Endoscopic rectal ultrasound
ISR	Intersphincteric APR
KRAS	Kirsten rat sarcoma virus
LARC	Locally advanced rectal cancer
LS	Lynch syndrome
LR	Local recurrence/pelvic recurrence
MMR	Mismatch repair genes
MRF	Mesorectal fascia
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stable
OS	Overall survival
pCR	Pathologic complete remission
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression-free survival
RC	Rectal cancer
RFS	Recurrence-free survival
RT	Radiotherapy
scRT	Short-course radiotherapy (5Gyx5)
TAMIS	Transanal minimally invasive surgery
TEM	Transanal endoscopic microsurgery
TME	Total mesorectal excision surgery
TNM	Tumour, node, metastasis
TNT	Total neoadjuvant therapy
TTR	Time to recurrence
TRG	Tumour regression grade

CONTENTS

1 INTRODUCTION

1.1 BACKGROUND

Colorectal cancer (CRC) is the third most common cancer type and the second leading cause of cancer mortality worldwide. According to statistics from the International Agency for Research on Cancer (IARC), an estimated 1. 8 million patients were diagnosed with CRC and 862, 000 died of CRC globally in 2018 [1]. Incidence is highly age-dependent and varies widely among less socioeconomically developed countries with the lowest values in Africa and the highest values in northern Europe and North America [1]. The disease is closely linked to western lifestyles [1]. Rectal cancer (RC) comprises approximately 1/3 of CRC cases. In Sweden, the annual incidence of RC during the period between 2012-2016 was 25/100, 000 for males and 17/100, 000 for females. This translates into absolute values of an incidence of 1200 RC for men and 800 for women per year [2]. During the same time interval, 5% of RC patients were younger than 50 years and 21% were older than 80 [3]. The annual mortality rate for RC in Sweden is estimated to be approximately 800 cases [3].

No single risk factor has been proposed as the aetiology for CRC. Correlations have been documented with age, hereditary genetic CRC syndromes, obesity, inflammatory bowel disease, and dietary factors such as high consumption of red processed meat and gender (increased risk for RC in males) [4-6].

1.2 ANATOMY

The definition of rectum is highly variable, and consensus is lacking regarding the exact proximal and distal borders of the rectum. The length of the rectum is also dependent on gender and the size of the patient [7].

In Sweden, the rectum is defined as the most distal 15 cm of the bowel from the anal verge, measured by rigid sigmoidoscope [8]. The rectum is usually divided into three parts, lower third, 0-5 cm from the anal sphincter, a middle section of 6-10 cm, and the upper third of 10-15 cm. The lower part of rectum ends at the anal sphincter complex, consisting of the internal and external sphincters. The internal anal sphincter can be viewed as a continuation of the muscularis propria of rectal wall. The external sphincter is composed of the inferior portion of the levator ani muscle, external sphincter muscle, and puborectalis sling. The most caudal border of the sphincter complex is defined as the anal verge at which the skin meets the anal mucosa. The surgical anal canal is defined as the region with the anal verge the caudal border and the levator ani as the cranial border.

The upper third of rectum is localized intraperitoneally while the lower 2/3 section is usually extraperitoneal, but this varies by individual and gender. The anterior peritoneum covers the upper 1/3 section of the rectum and the lowest part (above the uterocervical region in females and the seminal vesicles in males) makes a turn upwards with a V-shape configuration called the peritoneal reflection edge. Below the peritoneal reflection, the rectum is surrounded by a circumferential fatty sheath, called the mesorectum, that contains draining lymphatic channels and nodes, nerves, branches of a

rectalis superior, and the venous system. The mesorectum demonstrates variable thickness along its course with the thickest part in the upper rectum, posteriorly and laterally, but tapering caudally toward the lower rectum, exposing the distal rectum as a tube in continuity with the internal anal sphincter. This could provide a partial explanation for the higher LR rates in low RC as minimal extension outside the rectal wall in the distal segment results directly in overgrowth to adjacent structures with difficulty in achieving radical resection. The mesorectum is enclosed by a fascia called the mesorectal fascia (MRF) which is an important anatomical landmark in rectal oncology as surgical dissection planes are along the MRF.

1.3 PATHOLOGY

The rectal wall consists of five layers including (from inside out) 1: Mucosa lined by columnar epithelium, 2: Muscularis mucosa, 3: Submucosa, 4: Muscularis propria composed of inner circular and outer longitudinal muscle layers, 5: Serosa.

The most common type of RC is adenocarcinoma that has its origin in mucosal columnar epithelium organized in a glandular formation. In some cases, a cribriform formation with central necrosis can be found. The mucinous adenocarcinoma subtype, in which cancer cells secret extracellular mucin, comprises at least 50% of these tumours. This subtype is often associated with microsatellite instability. Mucinous changes can be observed in some tumours because of radiochemotherapy where large amounts of acellular mucin can be seen on histopathological examination. In this case, it is not classified as a mucinous cancer. In 3%-4% of CRC cases, other non-epithelial cell types in the rectal wall can become malignant and other cancer types, such as neuroendocrine cancers, sarcomas, and lymphomas may arise.

1.4 STAGING

Tumour, node, metastasis (TNM) staging is the most commonly applied cancer staging system where T describes the depth of tumour invasion into the rectal wall, N refers to metastasis to regional perirectal lymph nodes, and M designates distant metastasis. Although indispensable, TNM does not incorporate every crucial aspect of tumour extension, such as involvement of MRF which has a known impact on patient oncologic outcome. The prefix “c before T, N, and M indicates clinically-defined staging, usually defined on imaging while the prefix “p” is used for TNM staging on histopathological specimens in patients with surgery performed upfront without any preoperative treatment. The prefix “yp” is applied for histopathological staging in patients who undergo pre-operative oncologic treatment.

Table 1. TNM classification of CRC according to AJCC 8th edition

Category	Description
T category	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invading submucosa
T2	Tumour penetrating muscularis propria
T3	Tumour penetration into subserosa and perirectal fat tissue
T4	
a	Tumour invades surface of visceral peritoneum
b	Tumour directly invades adjacent organs and structures
N category	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis or tumour deposit
N1	
a	Metastasis in 1 lymph node
b	Metastasis in 2–3 lymph nodes
c	Tumour deposit(s) in the subserosa, mesentery or peritonealised perirectal tissue
N2	
a	Metastasis in 4–6 lymph nodes
b	Metastasis in 7 or more lymph nodes
M category	
M0	No distant metastasis
M1	Distant metastasis
a	Metastasis confined to one organ or site (non-regional lymph nodes, liver, lungs)
b	Metastasis in more than one organ/site or in the peritoneum

1.5 HEREDITARY COLORECTAL CANCER

It is estimated that 20%-30% of CRC cases are hereditary with 5%-10% related to known genetically well-defined hereditary CRC syndromes [9]. Hereditary CRC syndromes are subclassified as nonpolyposis colorectal cancer syndrome (HNPCC) or polyposis syndromes which are characterized by multiple polyps in the colon and rectum. Familial adenomatous polyposis (FAP) and its attenuated form (AFAP) comprise the most common diagnosis in the polyposis group which accounts for 0. 5%-1% of all CRC cases [9]. HNPCC is the most common hereditary nonpolyposis CRC syndrome which is implicated in 3%-5% of all CRC cases. These hereditary CRC syndromes are characterized by synchronous or metachronous CRC and extracolonic manifestations [9].

HNPCC, which is synonymously called Lynch syndrome (LS), is an autosomal dominant syndrome with high penetrance, characterized by CRC and other malignancies with early age of onset and metachronous extracolonic cancer diseases [10]. The average age for LS patients with a CRC diagnosis is 45 years in comparison to 72 years in the general population [10].

Lynch-associated CRC presents with characteristic features such as poor differentiation, mucinous adenocarcinomas exhibiting tumour-infiltrating lymphocytes, and Crohn-like inflammatory reaction [11]. Furthermore, the tumours have a tendency toward a more local aggressive behaviour with infiltration of adjacent organs such as the peritoneum and abdominal wall and, at the same time, a lower tendency for nodal and distant metastasis [12]. Lynch CRC has a predilection to be right sided with 70% of tumours proximal to the splenic flexure [11].

LS results from an inherited germline mutation in one allele of one of the DNA mismatch repair genes (MMR genes) [13]. MMR proteins function as part of the DNA repair complex and loss of MMR protein function results in defective DNA repair of mismatched base pairs during DNA replication. These defects are inherited to daughter cells and consequently lead to repetitive DNA sequences called microsatellites which lead to unstable DNA structures (MSI-high/Microsatellite instability). Mutations in MMR genes most commonly affect MLH1 and MSH2 and less frequently affect MSH6 and PMS2 [14]. A small proportion of LS patients have germline deletion of part of the EPCAM gene upstream of MSH2 with MSH2-methylation and silencing as a result [15]. The cancer panorama varies between different MMR gene mutations as age of onset [10]. Sporadic non-hereditary mutation has been described [16].

The average cumulative lifetime risk for CRC in LS has been reported to be up to 47% for males and 37% for females by 70 years of age depending on MMR mutation, with higher values for MSH2 [17].

The incidence of extracolonic cancer is increased in LS patients, in particular endometrial cancer, small bowel cancers, and central nervous system (CNS) tumours [16].

High cumulative lifetime risk for malignant disease necessitates early identification of pre-disposed mutation carriers and Lynch families. Diagnostic inclusion criteria and clinical guidelines have been established with the aim of identification of patients with germline mutations in MMR genes and HNPCC. The two major guidelines, Amsterdam II and revised Bethesda, have been applied for this purpose [18, 19] (Table 2). Tumours

from CRC patients that meet inclusion criteria according to Amsterdam II or revised Bethesda guidelines can be further analysed by immunohistochemistry to detect dysfunctional MMR proteins or by PCR to detect MSI high tumours. Failure rates for missing HNPCC cases with the Amsterdam criteria and the Bethesda guidelines are estimated to be approximately 50% and 30%, respectively, which necessitates utilization of other diagnostic measures, such as immunohistochemistry and PCR [20]. To avoid the low sensitivity of clinical guidelines, some researchers advocate for universal application of immunohistochemistry and/or PCR on all CRC and endometrial cancer specimens diagnosed before age 70 [21-23]. PCR is a highly reproducible diagnostic method that detects MSI-high tumours with 93% sensitivity [24]. Approximately 15%-20% of sporadic CRC cases demonstrate MSI-high levels on PCR which is the result of epigenetic hypermethylation of the MLH1 promotor [25]. Sporadic MSI-high tumours often harbour *BRAF* mutations, distinguishing them from Lynch-associated CRC in which *BRAF* mutation occur rarely [26]. Gene sequencing of MMR genes in blood lymphocytes is the most sensitive diagnostic method for verifying mutations in MMR genes, particularly in relatives without cancer history.

Table 2: Amsterdam II and revised Bethesda guidelines for diagnosis of LS

Amsterdam II Criteria (*All the criteria should be present)*	**Revised Bethesda Criteria** *(At least one criterion is required)*
There should be at least 3 relatives with HNPCC related cancer (CRC, endometrial cancer, cancer of stomach, duodenum, and urothelial cell cancer	Debut of CRC in a relative <50 years of age
At least one should be a first-degree relative of the other two	Second synchronous or metachronous CRC- or HNPCC-associated extracolonic cancer
At least one patient with cancer diagnosis before age 50	Pathologic features of MSI-high in tumours (lymphocyte infiltration, mucinous/signet ring cell differentiation, Crohn's-like inflammatory reaction
Involvement of at least two generations	Pathologic features of MSI-high in tumours (lymphocyte infiltration, mucinous/signet ring cell differentiation, Crohn's-like inflammatory reaction
FAP diagnosis must be excluded	CRC- or HNPCC-associated cancer in two or more first- or second-degree relatives at any age
	Second HNPCC-associated extracolonic cancer

1.6 p53

One of the most common genetic alterations in human cancers is mutation in the tumour suppressor gene p53 that occurs in more than 50% of cases. The p53 protein is activated upon cellular stress signals such as oncogene activation, hypoxia, radiation, or DNA

damage, and integrates these stress signals into a multitude of downstream responses such as cell cycle arrest, DNA repair, senescence, apoptosis, stem cell reprogramming, metabolism, and cell death [27, 28]. The p53 protein exerts its functions mainly as a transcription factor which regulates expression of hundreds of target genes as a response to cell stress [27]. Mutations in the TP53 gene lead not only to loss of its cell-protective function but also confer gain-of-function changes with oncogenic properties promoting carcinogenesis [29].

The TP53 gene encoding p53 is located on the short arm of chromosome 17. At least 12 isoforms have been identified as a result of alternative promotors and splicing [30]. The expression of isoforms is tissue-dependent with different cells and tissues expressing different isoforms [30]. Different isoforms function in a tissue-specific manner meaning the same stress can activate different p53 downstream effectors in different cells and organs [30].

The heterogenous outcomes upon p53 activation are not completely understood. High-throughput data has demonstrated 3509 potential p53 target genes in different cell lines [31]. Evidence indicates that p53 interactions with other proteins, temporal dynamics, and patterns of activity, together with DNA binding properties are determinants for cellular outcomes [32].

The p53 mutation in CRC demonstrates an age-dependent prevalence and is more common in patients under the age of 40 [33]. P53 mutation rates differ between the right and left colon with the distal colon and rectum more often harbouring mutations than the right-sided colon (45% mutation rate in left-sided and rectum vs 34% in the right colon) [34]. This has been explained by the abundance of anaerobic bacteria in the distal colon that produce gallic acid that prevents mutated p53 inhibition of the WNT proto-oncogene signalling pathway [35]. In the model of CRC progression from normal epithelium to adenoma and cancer proposed by Vogelstein, p53 is responsible for progression from high grade dysplasia to cancer [36].

There is also some evidence pointing to associations between the p53 gene and drug resistance against some cytostatic therapies such as 5-fluorouracil, gemcitabine, and the anti-epidermal growth factor receptor (EGFR) antibody cetuximab [37, 38]. This association between drug resistance and mutated p53 has also been demonstrated in vitro and in mouse models [39]. Zaidi examined the impact of mutations in 205 genes, including p53, in 2015 patients with CRC and found a worse CRC-specific survival for cases with p53 mutations (HR=1. 53, 95% CI 1. 21-1. 94) [40]. Sclafani examined the deleterious effect of *KRAS* and p53 mutation in 210 locally advance rectal cancer (LARC) patients [41]. The mutation rates were 44% and 60% for *KRAS* and p53, respectively [41]. Mutations in p53 were associated with higher rates of MRI-detected extramural vascular invasion (cEMVI+) on baseline MRI-staging (78% vs 65%, p=0. 04) and a non-significant but numerically different pCR rate between wild-type and mutated p53 (17% vs 9%, p=0. 08), and a tendency for worse 5-year progression-free survival (PFS) for mutated (HR 1. 59, p=0. 06). Even worse PFS was observed if both *KRAS* and p53 were mutated (HR 1. 75, p=0. 02) [41].

In a meta-analysis based on 1830 cases, Chen et al. found an association between p53 state and pCR after preoperative treatment with higher probability for patients with wild-type p53 to attain pCR (risk 2. 78, 95% CI 1. 40-5. 50, p=0. 003) [42].

Results from other studies indicate that p53 mutations might even have an impact on the biological behaviours of CRC such as increased invasiveness and metastatic potential. In proximal CRC, p53 mutations has been correlated to lymphatic invasion and, in the left colon and rectum, tend to have enhanced capability to invade lymph nodes and for vascular invasion [26].

The pivotal role of p53 in cancer development and progression has made it an attractive target for pharmacologic therapy of cancer. In the last decade, we have witnessed an array of small molecules that have been developed to restore p53 function in mutated cells mainly through restoration of wild-type structural conformation and DNA binding ability [43-45].

1.7 IMAGING

Accurate clinical staging of rectal tumours by imaging is crucial for optimal treatment planning of RC patients. Immense developments in imaging technology have been witnessed in recent decades, resulting in identification of prognostic factors for recurrence and curability in early-stage disease. Magnetic resonance imaging (MRI) plays a pivotal role in pre-treatment staging and is a valuable tool for therapy assessment, particularly if a pCR state has been achieved. Additionally, MRI-defined tumour regression grade (TRG) provides information about responders vs non-responders with predictive value for patients' OS. Imaging modalities available for diagnosis, local and systemic staging, and evaluating response include MRI, computed tomography (CT), endoscopic rectal ultrasound (ERUS) and positron emission tomography (PET-CT).

1.7.1 Endoscopic rectal ultrasound (ERUS)

ERUS with a rigid probe is used mainly for low early rectal tumours. The value of ERUS is more limited for tumours in the upper rectum or bulky, obstructive, and advanced tumours [46]. Patel et al. reported ERUS accuracy for T and N staging of approximately 70% and 65%, respectively [47]. ERUS has clear limitations that has restricted its role in clinical staging for rectal tumours. It has small field of view that limits assessment of the whole mesorectum, extramural deposits, EMVI, nodes not in proximity of primary tumours, and whether MRF is involved [48, 49].

1.7.2 Computed tomography (CT)

CT is a structural and anatomical technique widely used in clinical staging and restaging of RC patients with the aim of detecting distant metastasis in the thorax and abdomen. With the advent of multidetector CT with ultrathin slices, its accuracy for local staging of primary tumour T and N stage has increased to 86% and 84%, respectively [48, 50]. The performance of CT for detecting the depth of invasion into the rectal wall stage is more accurate for advanced T stage than early T1/T2 cancers. Previous studies have demonstrated a low 50% sensitivity for CT to predict the critical question of whether the MRF is involved [51]. A study by Ippolito compared MRI with multiplanar reconstructed CT in 91 RC patients and reported a sensitivity and specificity of 87% for CT in assessing

positivity of the MRF [52]. In contrast to ERUS, CT can provide visualization of EMVI [53].

1.7.3 Magnetic resonance imaging (MRI)

High spatial MRI has evolved into a standard modality for RC in the context of primary diagnosis, local staging, re-staging after pre-operative oncologic treatment, and detection of pelvic recurrence. MRI enables stratification of local tumours and identifies high risk features beyond T and N stage that can influence patient prognosis and survival such as MRF and EMVI, information which defines the treatment plan for RC patients [54, 55]. In a meta-analysis of 21 studies, MRI was demonstrated to have an 87% sensitivity (CI 81-92) and 75% specificity (CI 68-80) for T stage [56]. In the Mercury Study, MRI and histopathologic assessment of T stage in patients who underwent surgery upfront was considered to be equivalent to within 5 mm, especially for T3 tumours [57]. MRI has also demonstrated high accuracy for identifying the potential distance from the tumour to the MRF to within 1 mm [58, 59]. The Mercury prospective study demonstrated a 93% accuracy of MRI for involved CRM [44].

Radiologic assessment of nodal staging is less accurate than T category and MRF involvement due, in part, to variability of criteria applied for pathologic nodes. Previous studies have not demonstrated a reliable correlation between lymph node size and metastatic growth [48, 56], nor any cut-off that could be defined to assess nodes as pathologic or negative. Morphologic criteria, such as heterogenous MRI signal and irregular borders, need to be added to size criteria for more precise nodal staging [60].

EMVI, defined as tumour mass in a blood vessel beyond the muscularis propria, has been introduced as a high-risk feature for systemic recurrence and its association with disease-free survival (DFS) and distant metastasis [40].

Usually, RC patients requiring neoadjuvant treatment undergo restaging with MRI to assess response to therapy. A grading system based on qualitative evaluation of signal intensity in the tumour, the modified Mandard grading of tumour response, was the subject of investigation in the Mercury study [61] which confirmed the impact of tumour response for DFS and OS [62]. MRI-defined TRG 1-2 exhibits low signal intensity in the treated tumour with no evident residual tumour tissue, MRI TRG 3 demonstrates a dominant fibrotic outgrowing tumour mass, and TRG 4-5 is associated with minimal or lack of any response [47].

It is believed that both MRI and ERUS have lower accuracy for T stage determination after pre-operative treatment, declining to 50% [63]. Likewise, determining MRF positivity can be challenging and less accurate after neoadjuvant treatment with sensitivity and specificity of approximately 75% and 85%, respectively [64].

1.7.4 PET-CT

PET-CT combines structural-anatomical information (CT) with the functional-metabolic information from PET. Its role in primary and post (C)RT has yet to be defined. A meta-analysis addressed the value of PET-CT for assessment of mesorectal and pelvic lymph nodes. The pooled estimate of sensitivity was 42% and specificity was 88% [65]. In a

prospective study in high-risk low RC (MRI-predicted score 3-4, >5 mm extramural invasion, involved MRF and positive EMVI), PET-CT could detect synchronous distant metastasis in 20% of patients [66]. In the context of complete response to pre-operative treatment, a study by Zhang et al. demonstrated sensitivity, specificity, and positive predictive values of 78%, 66%, and 70% respectively [67].

1.8 SURGERY

Although we have experienced an evolution toward non-operative management for selected RC patients for 10-15 years, surgery remains the mainstay of curative therapy for RC. Historically, the first successful rectal resection was performed by Jacque Lisfranc in 1826. To avoid spillage and high rates of post-operative infections, an abdominal approach was introduced in 1874 and a combination abdominal and perineal procedure late in the 19th century. Results from these pioneering procedures were associated with high peri-operative and post-operative complications with LR rates of 80%, 3-year OS <15%, and an operative mortality rate of around 20%.

The ground for a more modern and radical surgery was laid down by Miles with his seminal publication in Lancet in 1908 as he proposed and designed a procedure called the abdominoperineal resection (APR) [68]. Introduction of APR indicated a deeper understanding of the natural history of RC with dissemination via the lymphatic system being mainly upward. The traditional APR consists of resection of the pelvic colon, proctectomy, mesorectum, lymphadenectomy up to the iliac bifurcation via an abdominal approach, and resection of the anus, sphincter complex, and levator ani through perineal approach. Practice of the APR concept resulted in a clear decrease in LR rates down to 30%. A later modified two-stage APR was followed by a synchronous one-stage perineal and laparotomy approach.

A major event in RC surgery was the application of total mesorectal excision (TME) [69]. Development of modern surgical resection had its origin in the recognition of both the importance of lateral circumferential resection margin for LR and a reappraisal of the distal resection margin. Quirke and colleagues reported a retrospective study with 52 RC specimens with positive lateral margins in 25% of cases of which 80% experienced LR [70]. This obstacle was addressed by TME.

The rationale for TME was exploitation of the MRF as the plane of dissection along the avascular perimesorectal, resulting in a cylindrical specimen consisting of the rectum, mesorectum harbouring lymphatic drainage of the tumour, and the MRF as an intact envelope. Heald published his results of TME applied to 519 patients resected between 1978 and 1997 in which he reported a LR rate of 8% and a 10-year disease-specific OS as high as 66% [71].

Meanwhile, a negative impact on quality of life in many resected patients related to discontinuity of bowel and dysfunctional neorectum resulted in successive shrinkage of the distal resection margin from the traditional 5 cm distal margins. Williams examined 50 potentially curative specimens and found that 90% had no or <1 cm intramural distal spread from the primary tumour [72]. The consequence was low anterior resection (LAR) procedures with less distal resection margin down to 1 cm without impact on LR or OS [73].

Radical surgical techniques can be classified into two main groups, sphincter-preserving resections and non-sphincter preserving procedures.

Sphincter preserving resections include LAR, ultra-LAR, and intersphincteric APR (ISR). Non-sphincter preserving procedures include traditional APR and extralevator APR (ELAPE).

A LAR technique with colorectal anastomosis implies sigmoidectomy and proctectomy, sparing the distal portion of the rectum and a colon J-pouch or with straight anastomosis. LAR with TME is viewed as the gold standard surgical technique for tumours in the upper-mid rectum with resection below the peritoneal reflection. The successive shrinkage in DRM has led to use of LAR for tumours in the lower rectum and resection down to the pelvic floor below the mesorectum with rectal mucosa within the functional anal canal (Ultra LAR with coloanal anastomosis). A frequent post-operative complication with impact on patient quality of life has been described as post-proctectomy syndrome (LAR syndrome) characterized by increased frequency, urgency, and clustering of bowel evacuation, incontinence, sexual and urinary dysfunctionality [74, 75].

Intersphincteric APR is a modified version of traditional APR for very low rectal tumours that does not involve the anal sphincter complex but requires resection below the tumour to assure a negative DRM. In this procedure, the surgeon exploits the space between the intersphincteric space, removing the internal sphincter while the voluntary external sphincter remains intact, a compromise between oncologic outcome and less impact on continence [76].

Extralevator APR addresses high local failure in traditional APR procedures. A decrease in positive CRM after APR has not paralleled the widespread application of pre-operative chemoradiation or TME surgery. Comparison to LAR demonstrates higher rates of positive resection margins, higher rates of local failure, and worse OS, in part due to lack of mesorectum at the distal section of the rectum [77].

Short-term oncologic outcome has been a subject of debate. A study published by West pointed to a reduction in positive CRM from 50% to 20% compared to standard APR and a decrease of intraoperative perforation from 28% to 8% [78]. Other studies confirmed the lower rates of involved CRM and perforation rate [79]. In contrast, a population-based study from Denmark including 554 patients did not observe reduced positive CRM nor perforation rates [80]. As for short-term outcomes, data on long-term ones are contrasting with regard to the advantage of ELAPE vs standard procedure [81, 82].

In recent years, we have witnessed efforts toward less invasive surgery in the RC surgical domain. Laparoscopic RC surgery has been shown to be associated with less pain, earlier return of bowel function, and shorter length of hospital stay [83].

Transanal endoscopic microsurgery (TEM) and transanal minimally invasive surgery (TAMIS) for local excision of rectal tumours have been developed for avoiding transabdominal surgeries for early RC, in combination with CRT for more advanced tumours and in frail patients. Although attractive, the oncologic outcomes have not been the subject of prospective randomized studies. Patient and tumour characteristics determine selection of RC for TEM/TAMIS. Low risk tumour features include early T1, node negative, <3 cm in diameter, low grade, without lymphovascular invasion, localized

in the lower rectum, and involving limited rectal wall circumference. A retrospective study by Borschitz demonstrated an 89% 10-year OS for patients with low-risk tumours, R0 resection with >1 mm marginal vs 49% for high-risk group. Finding of pathological high-risk feature should warrant consideration of TME or other complementary measures [84].

1.9 ONCOLOGIC PERSPECTIVES

1.9.1 Preoperative treatment

Radiation refers to emission and propagation of energy through space or a medium by means of waves and particles. If carrying sufficiently high energy, it results in ionization of target molecules in tissues and material (Ionizing radiation). Radiotherapy exploits the toxic effects of ionization in tissues to treat malign diseases. Ionizing radiation is generated through advanced linear accelerators and transmitted through energy packages (photons) to target tissue (External beam radiotherapy). In living organisms, the main target for ionizing radiation is DNA molecules. High energy photons interact with DNA, generating toxic free radicals from water molecules resulting in damage to cellular genetic machinery. In contrast to normal cells, cancer cells are characterized by high proliferative and mitotic activity with defective DNA repair resources. A single or double strand DNA break in tumour cells because of radiation with diminished ability to repair the damage and consequent cell death is the rationale for treating cancer with radiation therapy.

1.9.2 Pre- vs post-operative radiotherapy

The fact that historically, before TME was introduced, patients with RC experienced more local failure than colon cancer patients (and not infrequently as isolated events without simultaneous systemic relapse) resulted in the design of randomized trials to decrease pelvic recurrence rates. Early trials utilized post-operative radiotherapy (RT), chemotherapy (CTX), or a combination of the two post-operatively [85, 86]. The American R-01 3-armed randomized trial enrolled patients with T3/T4 or N+ RC to surgery, surgery and adjuvant CTX, and surgery plus post-op RT [87]. After a follow-up of 64 months, an overall improvement in DFS and OS was observed for surgery plus post-op CTX vs surgery alone. In the treatment arm with surgery plus RT, a marked reduction in locoregional failure was observed vs surgery (16% vs 25% for surgery alone). No effect on OS could be observed for the RT plus surgery arm [88]. The subsequent R-02 study randomized patients to surgery plus adjuvant chemoradiotherapy (CRT) vs surgery plus adjuvant CTX and confirmed the benefit in local control in the post-op CRT arm vs adjuvant CTX (8% vs 13% p=0. 02) [89].

These studies were performed in the pre-TME era and established the role of post-operative RT in decreasing LR rates in RC patients. By the late 1970s, the timepoint for delivering (C)RT was under investigation as some theoretical advantages could be postulated if oncologic treatment was delivered pre-operatively. These include down-sizing to improve resectability, sterilization of lymph channels draining the tumour,

better oxygenation in pre-op tissue which could enhance RT effects, and exclusion of the small bowel from the radiation field by native rectum pre-operatively. Several European trials examined the feasibility of pre-operative RT parallel with the American trials with continued focus on post-operative delivery of RT. This led to several ground-breaking trials conducted in Sweden with the invention of short-course RT (scRT) 5Gyx5 administered pre-operatively in one week and surgery immediately after [90-92]. Swedish trials demonstrated the feasibility of delivering RT pre-operatively and corroborated the benefit of RT for reducing pelvic recurrence. LR rates were significantly reduced with preoperative RT (12% preoperative RT vs 21% for post-operative RT, p=0. 02). In addition, they reported an OS benefit in the younger patient category, although the rate of distant failure was unaffected [92].

In the meantime, TME surgery gained ground and a diminished utilization of RT was anticipated as TME without RT showed a promising decrease in LR rates. This was the subject of a Dutch TME trial that included 1861 patients and confirmed a 50% reduction in LR after pre-operative ScRT followed by TME vs TME alone [93]. No effect on OS was observed in Dutch TME trial. The Dutch trial proved the value of RT and its beneficial role even when combined with TME surgery [93].

The German rectal cancer group CAO/ARO/AIO-94 investigated pre- vs post-op CRT in a randomized trial including 823 patients with advanced T3/T4 tumours or node-positive disease and TME resection [94]. The pre-operative group had significantly lower LR rates (6% vs 13% in the post-op group, p<0. 006). Patients in the pre-operative group experienced less acute and late toxicity than those receiving treatment post-op [94]. The German study was pivotal in establishing the role of oncologic treatment in the pre-operative setting.

1.9.3 Development of preoperative treatment schedules

Since RT became an integral part of RC treatment, several dosage schedules with/without concurrent CTX have been applied. During the past three decades, two RT courses emerged as the most dominant in clinical trial settings and were utilized in routine practice, scRT as introduced by the Swedish group and long-course RT up to 50-55 Gy, concurrent with administration of 5-Fluorouracil (5-FU)(CRT) [92].

scRT delivered in 5 Gy fractions over 5 consecutive days with surgery the week after and no concurrent CTX is currently applied. This concept has gained widespread acceptance, mainly in Northern Europe. CRT delivers much smaller fractions, daily 1. 8-2 Gy fractions for 5-6 weeks up to 45-55 Gy concurrent with a varying administration of 5-FU/capecitabine and delayed surgery 6-8 weeks after ending neoadjuvant treatment. CRT is popular mainly in the US, Germany, and southern European countries. The rationale behind concurrent CRT with administration of 5-FU/capecitabine is to potentiate the effect of administered RT.

Two comparative randomized trials have addressed the hypothesis of whether CRT was superior to scRT [95, 96].

The Polish rectal cancer trial compared scRT with immediate surgery the week after completing RT or CRT pre-operatively with 1. 8 Gy fractions in 28 fractions (total 50. 4 Gy) with concurrent 5-FU/leucovorin in the first and fifth week of RT [95]. Eligible

patients were T3/4 low rectal cancer. The study included 312 patients between 1999-2002. Patients who underwent CRT experienced significantly higher levels of acute grade III toxicity (18% vs 3%, p<0. 001) but no difference in late toxicities or post-operative complications was observed. Patients in the CRT group experienced more pathological complete responses (pCR) (16% in CRT vs 1% scRT). The observed higher levels of pCR in the CRT arm could be related to a longer delay from completing CRT to time for surgery. After a median of 4 years of follow-up, there was no difference between the arms for OS, DFS, LR, or late severe toxicity [95].

Another randomized study to compare scRT with CRT was the Australian TROG01. 04 trial with a similar design to the Polish study but limited to a more uniform patient group with T3 RC [96]. After a median follow-up of 5. 9 years, no significant differences between the two arms were observed for late toxicity, systemic relapse, or OS [97]. LR was higher in the scRT group compared to the CRT group but did not reach statistical significance (CRT 4. 4% vs scRT 7. 5%, p=0. 24). Similar to the Polish trial, pCR rates were higher for the CRT arm (CRT 15% vs scRT 1%, p<0. 001). A subgroup analysis demonstrated a large difference in pelvic recurrence for low tumours (<5 cm from the anal verge) between the two arms (scRT 12. 5% vs CRT 3. 2%, p=0. 21). This finding, despite falling short of statistical significance, resulted in the authors' conclusion that for bulky low RC, CRT would offer an advantage compared to scRT. As expected, a difference could be confirmed for pCR in CRT with delay vs scRT with immediate surgery (11. 8 vs 1. 8%, p=0. 001) [96].

No prospective randomized trial has compared scRT with delayed surgery with CRT, but retrospective population-based studies have demonstrated a significantly higher pCR rate for CRT than scRT with delayed surgery [98, 99]. As both schedules use delayed surgery, the higher pCR rate in CRT cannot be ascribed to differences in time interval from RT to surgery but a more efficient treatment for reaching pCR due to its higher cell-killing effect.

Results from the Polish and Australian trials could point to the advantage of a delay from the end of scRT/CRT to time for surgery as higher rates of pCR were observed in both studies. The Stockholm III randomized trial compared scRT with immediate surgery the week after, scRT with delayed surgery >4 weeks after completion of RT, and a long-course RT arm 2 Gy in 25 fractions without concurrent CTX [100, 101]. Published results after including 840 patients could not confirm any difference between the three arms for local or systemic relapse or OS. Similarly, no difference could be detected between the three arms when results from the three arms were pooled with the two-arm randomized groups. Patients in the scRT group with immediate surgery did not experience acute grade 3-4 toxicity in contrast to scRT with delay (6%) and long-course RT (5%). In the pooled analysis, 1% of subjects in the scRT group with immediate surgery experienced acute toxicity which was significantly lower than scRT with delayed surgery (1% vs 7%, p<0. 0001). However, scRT with immediate surgery had a higher rate of surgical (36% vs 28%, p=0. 03) and any post-operative complications compared to delayed surgery (53% vs 41%, p=0. 001) [102]. The observed pCR rate was 0. 3% in scRT with direct surgery, 10. 4% in scRT with delayed surgery, and 2. 2% in long-course RT with delayed surgery.

Although both the Polish and Australian trials demonstrated a higher pCR in the CRT groups, this could theoretically be ascribed to delayed surgery in the CRT arm while scRT was followed by direct surgery. The design and results did not clarify the role of chemotherapy in the CRT schedule. A French randomized phase III trial, FFCD 9203, addressed the subject by including 733 patients with T3-T4 NX middle-low resectable RC to receive either RT 45 Gy in five weeks (standard arm with surgery 3-10 weeks after termination of RT) or the same RT schedule combined with 5-FU and leucovorin 350 mg/m^2/d over five days, weeks 1 and 5 [103]. pCR was observed in 11. 4% in the CRT group vs 3. 6% of the only RT group, p<05. Five-year LR was significantly lower in the CRT group than the RT group (8. 1% vs 16. 5%, p<0. 05) while there was no difference in OS between the groups [103].

The same design was used in a randomized phase III Swedish trial for more advanced, non-resectable T3-4 RC or patients with LR [104]. The trial included 207 patients between 1996-2003. The RT was delivered either in a single dose as 50 Gy (control arm) or RT combined with 5-FU and leucovorin concurrent and 16 weeks postoperatively. A higher R0 resection rate was achieved in the CRT arm (84% CRT vs 68% RT, p=0. 009), as well as a higher pCR rate (16% vs 7%), improved cancer-specific survival (72% vs 55%, p=0. 02), and improved 5-year OS (66% vs 53%, p=0. 09) [104].

To further potentiate the CRT treatment, several studies have examined the addition of a second drug to 5-FU concurrent with radiotherapy, with the aim of enhancing the pCR rate. The German randomized phase III trial CAO/ARO/AIO-04 accrued 1265 patients with T3/T4 or node-positive RC [105]. The same pre-operative treatment schedule as CAO/ARO/AIO-94 was delivered in the standard arm, the investigational arm received 50. 4 Gy in 28 fractions concurrent 5-FU and oxaliplatin [105]. Furthermore, patients received 8 cycles of post-op FOLFOX. After a median follow-up of 50 months, a superior DFS was observed for the experimental arm (75. 9% vs 71. 2%, HR 0. 79, p<0. 03) and a higher rate of late grade 3/4 adverse events in the investigational arm was reported (25% vs 21%) [105].

The French randomized trial ACCORD 12 enrolled 598 patients between 2005-2008 to study pre-operative CRT with capecitabine and RT 50. 4 Gy compared to capecitabine plus oxaliplatin (CAPOX) plus 50. 4 Gy. At 3 years, no difference in clinical outcome was detected between the two arms [106, 107].

A recent development in trimodal treatment of RC has been incorporation of CTX upfront or after RT for LARC (Total neoadjuvant treatment=TNT). This addresses early treatment of micrometastases by reducing delays in delivering CTX and better tolerability pre-operatively as recovery after RC surgery for many requires longer times, affecting patient compliance for post-operative CTX. Another theoretical advantage of this approach could be better oncologic outcomes in the form of higher tumour response, enhancing the probability of pCR and sphincter-preserving surgery [108].

Two phase II multicentre international European randomized trials, EXPERT and EXPERT-C addressed the question of sequential treatment CTX followed by CRT before TME surgery in poor risk RC groups [109, 110]. In the Expert-C group, the investigational arm received, in addition to CAPOX, cetuximab [85]. Pooled analysis of EXPERT and EXPERT-C with 269 high-risk RC patients treated with CAPOX demonstrated excellent compliance rates (91% for CAPOX and 88% for CRT) and an acceptable toxicity profile.

Five-year PFS and OS were 66% and 73%, respectively, and R0 resection was achieved in 98% [110].

Results from the EXPERT and similar parallel studies were an impetus to further strengthen the role of CTX in pre-operative trimodality sequential treatment at the expense of CRT exchanged with scRT [111, 112]. Phase III trials investigated scRT with consolidating CTX vs CRT. A Polish study enrolled 515 patients randomized with an investigational arm with scRT followed by 3 cycles of FOLFOX vs CRT with addition of oxaliplatin in clinical T4 or fixed T3 rectal cancer [113]. Initial publication after 3 years of follow-up demonstrated a superior OS for the investigational arm with 9% difference vs the control arm which did not reach statistical significance (HR 0. 90 CI 0. 7-1. 15, p=0. 38.) [113]. An update after 8 years of follow-up was published in which the difference in OS had disappeared [114]. In the last decade, this field has had increasing enthusiasm for applying pre-operative TNT based on assumptions of reduced systemic recurrence rate and better compliance to deliver the whole regimen before surgery with a more fit patient as previous post-operative adjuvant trials have suffered low accrual because of post-operative complications and slow recovery. [115-118]. The delivery of the whole regimen of oncologic treatment preoperatively also has the advantage of shortening the time for patients with mid-high RC in need of a post-operative stoma.

Recently, results from one phase II and three phase III trials have been published [119-122]. In the RAPIDO trial, treatments included the TNT arm with scRT followed by CAPOX before TME surgery in the experimental arm vs CRT before TME surgery and optional postoperative 8 cycles CAPOX, in the standard arm. With 920 patients included, a higher pCR rate of 28% was noted in the experimental arm compared to 14% in the standard arm, indicating superiority of TNT in achieving pCR [123]. After a median follow-up of 5. 4 years, local failure was higher in the experimental arm, 10% vs 7% in CRT group (p=0. 038) but distant metastasis rate was reduced in the TNT arm (23% TNT arm vs 31% CRT, p=0. 011) [123]. The observed benefit in less distant metastasis rate could not be translated to 5-year OS (HR 0. 92, 0. 87-1. 25, p0. 59) [121].

UNICANCER PRODIGE 23, a French phase III TNT trial, was designed differently with induction chemotherapy with triplet FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin) and CRT instead of scRT, a clearly more intensive treatment than RAPIDO [120]. A total of 461 patients were included in the trial. The investigational arm was treated with 6 cycles of FOLFIRINOX followed by CRT and TME surgery. Patients received 3 months of postoperative CAPOX/FOLFOX. The control arm started with CRT before TME surgery and 6 months CAPOX/FOLFOX. Three-year DFS improved by 7% in the TNT arm (75% vs 68%, p=0. 03) [120].

OPRA, a prospective phase II randomized trial from Memorial Sloan Kettering center was published recently [124]. As with PRODIGE 23, they selected CRT combined with either induction (IN-CRT) or consolidation (CRT-CNCT) FOLFOX for 4 months and TME surgery or watch and wait if CCR was achieved. The trial used a novel endpoint, 3-year TME-free survival. With 324 patients included, there was no difference in 3-year DFS, 76% for both arms. There was a difference in 3-year TME-free survival in favour of consolidation chemotherapy (53% vs 41% for IN-CRT). One conclusion could be that there was greater efficacy with consolidation chemotherapy, but another possible explanation could be that there was a longer interval between termination of

CRT to surgery in the consolidation arm than in the IN-CRT arm. (67% vs 67%) finding an answer about superiority of induction vs consolidation chemotherapy.

Some trials have been concluded or are ongoing to test omission of RT from the preoperative treatment schedule as there is a belief that long-term toxicity doesn't balance the very low local failure risk with introduction of TME surgery. Usually, these trials are restricted to tumours with less advanced features such as distal border 5 cm > above anal cT2-3 No and distance to MRF >2 mm verge. The large randomized American PROSPECTIV trial is ongoing (NCT01515787). The Chinese FOWARC phase III randomized trial in 495 patients randomized 1:1:1 in three arms, CRT, CRT (concurrent 4 cycles single 5-FU+leucovorin concurrent RT 50. 4Gy) and 7 cycles postoperative 5-FU vs CRT+FOLFOX (as arm A but with addition of oxaliplatin) or 4-6 cycles FOLFOX followed by surgery and postoperative FOLFOX x 6-8. The 3-year DFS was insignificantly improved in FOLFOX-CRT, 77% compared to 73% for CRT and 74% for the FOLFOX arms. No differences between groups were noted for local failure, around 8%. pCR rates were 27. 5% in FOLFOX-RT, 14% with CRT, and 6. 6% for FOLFOX without RT [125].

Other TNT trials have been conducted and recently published results [120, 121, 124, 126].

1.9.4 Time interval to surgery

A topic that has been a subject for intense debate is the time interval from termination of (C)RT to time for surgery. No consensus has been reached yet about this topic. Several retrospective studies have concluded that a longer interval, >8 weeks, preferably 10-11 weeks, between the end of (C)RT and the timepoint for resection has been associated with higher pCR rates [127-129].

The Lyon R90-1 randomized trial was the first to investigate the longer interval between completion of pre-operative treatment and timepoint for surgery (<2 weeks vs 6-8 weeks) [130]. Results from R90-1 showed that the longer interval was associated with a higher rate of downstaging and pCR/near-pCR (26% vs 10. 3%, p=0. 0054) and tumour response rate (71. 7% vs 53%, p=0. 007). A 15-year update concluded that there was no difference in local failure rate or OS between the two arms [130].

Another French randomized phase III trial, GRECCAR -6, compared the effect of longer interval, 7 vs 11 weeks, on pCR rate and surgical outcomes [131]. A total of 265 patients were included, the pCR rate was equal in both groups (7 weeks 15% vs 11 weeks 17. 4%, p=5983). Morbidity and worse quality of TME surgery was significantly more frequent in the 11-week arms (p=0. 0156) [131].

A comprehensive meta-analysis including 13 studies and 19, 652 patients was published in 2018 [132]. This review demonstrated that pCR was significantly increased if the waiting period until resection was >8 weeks vs <8 weeks (Risk ratio 1. 25, CI 1. 16-1. 35, p>0. 0001). The other clinical parameters DFS, OS, local and systemic relapse were similar between the two groups. The same conclusion was reported in another meta-analysis including 3584 patients with resection interval 6-8 weeks vs control group [133]. A significant difference in pCR rate still favoured the extended interval (19. 5% vs 13. 7%, p<0. 0001) [132].

In conclusion, we have witnessed a growing body of evidence favouring an extended interval between completion of (C)RT and resection with 8 weeks as a reasonable compromise.

1.10 TUMOUR REGRESSION GRADE

Since the introduction of pre-operative oncologic treatments in rectal cancer and to determine the spectrum of responses on histological examination of the resected specimen, efforts have been made to develop a system for classifying variations in treatment effect. Tumour regression grade is a way to stratify the degree of response in the tumour measured as residual viable tumour cells versus regressive changes (fibrosis) in a surgical specimen. Several classification systems, tumour regression grading (TRG), have been developed based on the number of regressive changes on histopathological investigation of the primary tumour specimen [134-137] (Table3).

Common to all these systems is assessment of residual viable tumour cells against regressive changes in the form of fibrosis. Fibrosis has demonstrated higher reproducibility in comparison to other infrequent changes such as degree of inflammatory infiltration in the tumour or mucin pools. Different TRG systems have shown variation in inter-observer agreement and lack of consistency between pathologists [138, 139].

A German study applied Rödel's TRG classification for assessment of correlations between different TRG groups for patients included in the AIO/ARO-CAO 94 randomized trial and found a significant correlation between TRG grade and rate of distant metastasis and DFS [140]. On multivariate analysis, only TRG and residual lymph node metastasis were predictive of distant metastasis. Several other studies confirmed correlations between TRG levels and oncologic outcomes [141].

Pathologic complete remission (pCR) is defined as no viable tumour cells in the resected specimen on histopathological examination and the site for primary tumour is covered by fibrotic reaction. Previous studies have demonstrated a strong correlation between pCR and OS [142, 143].

Table 3. TRG systems to evaluate neoadjuvant treatment response in resected RC specimens

Descriptive	Mandard	AJCC	Rödel	Dworak
Complete	TRG 1 No residual cancer cells	TRG 0 No residual cancer cells	TRG 4 Complete regression	TRG 4 No viable tumour cells detected
Subtotal	TRG 2 Single rare cancer cells	TRG 1 Single cell or small groups of cells	TRG 3 Fibrosis >50% of tumour mass	TRG3 Scattered tumour cells in the space of fibrosis with/without acellular mucin
Partial	TRG 3 Fibrosis outgrowing residual cancer	TRG 2 Residual cancer with desmoplastic response	TRG 2 Fibrosis 25%-50% of tumour mass	TRG 2 Predominantly fibrosis with scattered tumour cells (easy to find)
No regression	TRG 4 Residual cancer Outgrowing fibrosis	TRG 3 Minimal evidence of tumour response	TRG 1 Fibrosis <25% of tumour mass	TRG 1 Predominantly tumour with significant fibrosis
	TRG 5 Absence of regressive changes		TRG 0 No regressive changes	TRG 0 No regression

2 AIMS OF THE THESIS

Study I: To find new natural substances with preferential cytotoxic effect on cancer cells carrying mutated p53 gene.

Study II: To explore proportion of non-colonic, non-endometrial cancer diagnosis in Swedish Lynch families in relation to general population.

Study III: Examination of baseline clinical, imaging and laboratory parameters in predicting pCR in RC patients treated with one of three commonly used preoperative treatment schedules.

Study IV: Primary aim was to explore the strength of NAR score for oncological outcomes TTR, CSS and OS treated with scRT, CRT and scRT/CRT+CTX both for all patients and separately for each preoperative treatment. Secondary aim was to improve NAR score prognostic strength by combining with other imaging and histopathologic prognostic parameters.

3 MATERIAL AND METHODS

3.1 STUDY I

3.1.1 Materials

1. Cell lines

1) Osteosarcoma Saos-II -/- (p53null=No p53) and Saos-II-His273 (harbouring Tet-Off construct with mutated p53 at residue His 273).
2) Lung cancer adenocarcinoma H1299-/-(p53null) and H1299-His 175.
3) Colon adenocarcinoma HCT116-/-(p53null), HCT116+/+ (wild type), HCT Trp116248/- (mut Trp248 and p53null) and HCT116 Trp248/wt (wild type and mutated at Trp248).

2. Experimental substances (natural extracts)

1) N37063 (terrestrial plant, Asteraceae B. Ramiflora).
2) C3483 (marine invertebrates, Demospongiae Thorectidae Ircinia).
3) N12727(terrestrial plant, Flacourtiaceae Flacourtia indica).

3.1.2 Methods

Flow cytometry (FACS): A technology for rapid multiparametric analysis of particles and intracellular/extracellular cell characteristics, identification of cell subpopulations in a heterogenous cell suspension and measuring cell size. In addition, cell characteristics were assessed by using different fluorescent labels. In this study, two different FACS protocols were applied: ***Propidium Iodide FACS(PI-FACS) and CaspaTaq-FACS*.**

PI-FACS is based on the principle that cells with surface integrity and intact cell membranes exclude PI from entering cells while apoptotic cells with damaged cell membranes take up the fluorescent dye and the levels of fluorescence captured by the FACS machine reflects the levels of cell death.

CaspaTaq-FACS is a fluorescence-based assay that utilizes the principle of the activation cascade of caspase proteolytic enzymes as a central component of the apoptotic process in cells which ultimately results in activation of caspase 9 which is detected by fluorescein in the assay.

Fractionation: A screening procedure used to separate plant extract mixtures into organic hydrophobic, interphase, and aqueous fractions before isolation of active compound(s).

Growth suppression assays: Applied to measure cell proliferation and viability. WST-1 (Roche, Stockholm, Sweden) is a spectrophotometric quantification method to detect cell proliferation by measuring non-radioactive labelling of DNA content in cells. As

cells proliferate and increase their DNA synthesis, they incorporate more [^{3}H]-thymidine into their DNA and this can be quantified by spectrophotometry.

Glutathione-based depletion of substance N37063: To target putative active SH-group and its role in eliciting apoptosis in cells treated with substance N37063, we performed a glutathione-based depletion of SH-groups in N37063.

Immunohistochemistry: Cells were treated with test substance in different concentrations according to study design, fixed, and treated with primary antibody followed by addition of secondary fluorescent antibody and microscopic interpretation.

In silico screening of NCI library: Computational models applied for predicting toxic and pharmacological effects of substance/drug homologues to known substances.

Real-time reverse transcription-polymerase chain reaction (RT-PCR): A quantitative laboratory method used to detect expression of target sequences/genes and measure RNA. The principle of RT-PCR is based on reverse transcription of RNA into cDNA, multiple thermal cycles with exponential increase in amplicons that are visualized continuously by increasing fluorescence emissions.

TransAm P53: ELISA-based assay to detect DNA-P53 binding and quantification of transcription factor activity. A manufacturer-provided standard curve control was used for interpretation of results and to quantify specific P53-DNA binding.

Western Blot (=Immunoblotting): A semiquantitative analytical method used for detection and visualization of specific proteins in protein mixtures extracted from cells. The procedure is based on using gel electrophoresis and separation of proteins in a mixture according to size and weight.

3.2 STUDY II

3.2.1 Patients

This was a registry study of Swedish families with verified LS under follow-up by Departments of Clinical Genetics at university hospitals in the country. For this study, 5 of 6 Swedish university hospitals participated, providing full pedigree information, gender, cancer diagnosis, and age at onset. At least three consecutive generations were included for each family pedigree.

3.2.2 Statistics

The cohort was stratified for age, gender, mutated gene, and primary cancer(s). The relative proportion of different primary cancers in the general population was retrieved from national health board records (Socialstyrelsen) for two separate timepoints, 1970 and 2010 and compared with the relative proportion of each cancer type in the LS population. The distribution of cancer diagnoses was weighted by age and sex in relation to mutated MMR gene in control and target cohorts. Confidence interval (CI) was

calculated for both populations by using binomial distribution and a relative proportion of primary cancer diagnosis in both groups compared.

3.3 STUDY III AND IV

3.3.1 Patients

For this study, we retrospectively collected a cohort of consecutive patients with non-metastatic RC who received, with curative intent, one of the three commonly used pre-operative oncologic treatments and delayed surgery ≥4 weeks after termination of RT. The study cohort itself consisted of two separate independent cohorts, the first from Uppsala-Dalarna with patients treated between January 1, 2010, to December 31, 2018. The second cohort consisted of patients from Stockholm County diagnosed in the same way between January 1, 2006, and December 31, 2016. All patients were followed-up for recurrence and survival in line with national guidelines. Information concerning recurrence and survival was updated on March 24, 2022, before definitive data analysis. Several patients in each cohort were included and treated in previous randomized trials Stockholm III, EXPERT-C, and Rapido [100, 110, 121]. The same cohort in study III was used as a study cohort but the cohort from Uppsala-Dalarna was updated with inclusion of additional patients treated until December 31, 2020. The last date for follow-up was October 10, 2022. No patients were lost to follow-up.

The pre-operative treatment was delivered according to one of three schedules:

a) **scRT:** short-course 5Gyx5 in one week and delayed surgery.

b) **CRT:** Concurrent chemoradiotherapy, 1. 8Gyx25 or 2Gyx25 concomitant with capecitabine 825 mg/m^2 twice daily, days 1-38 or 900 mg/m^2 on RT days followed by delayed surgery.

c) **scRT/CRT+CTX=TNT**: Either scRT or CRT with induction or consolidation chemotherapy.

Stockholm III was a randomized trial with 3 arms A: 5Gyx5 and surgery following week, B:5Gyx5 and delayed surgery>4 weeks after completion of RT and C:2Gyx25 and delayed surgery>4 weeks. Only patients in arm B in Stockholm III were included in this study.

Patients included in EXPERT-C, a phase II randomized multinational trial, received four cycles of capecitabine and oxaliplatin (CAPOX) followed by CRT, TME surgery and an additional 4 cycles of CAPOX post-surgery (The experimental arm received treatment with cetuximab without stratification for wt/mut *KRAS*).

The RAPIDO trial was designed as a two-arm phase III multinational trial that included LARC with high-risk features on MRI staging. Patients were treated with either CRT (control arm) and post-operative CAPOX for 6 months vs scRT followed by 6 cycles CAPOX before TME surgery (experimental arm).

3.3.2 Statistics studies III+IV

AUC (Area under curve): Same as Receiver Operating Curve (ROC), used to evaluate performance of statistical predictive model and distinguishing probability for an event. AUC with value 1 indicates perfect model while AUC=0. 5 is associated with random probability.

X^2 test (Chi-squared test): non-parametric hypothesis testing for categorical variables to find associations between variables.

Binary logistic regression: Applied when dependent variable is of categorical nature but presented as numerical value. It is used for prediction modelling and goodness of fit between dependent and independent variables.

Cox regression model (Cox proportional hazards model): Multivariate regression model to detect association between predictive variables with time-to-event as a dependent variable.

Kaplan-Meier survival estimates: Applied for estimating probability of time-to-event variables (such as OS, TTR, OS) based on single binary predictor.

Only Study III

Mann-Whitney U-test: non-parametric test to compare means from two independent groups for categorical parameters.

Interaction analysis: To discriminate the effect of several independent variables on a dependent variable in a regression model.

Only Study IV

AIC (Akaike information criterion): Applied to assess the predictive strength of statistical models. Lower AIC value is associated with stronger predictive model.

C-index: Similar to AUC and ROC, applied to discriminate performance power of a predictive statistical model.

Forward modelling: A stepwise regression modelling adding stepwise predictive variables with the aim of finding the model with best fitness.

Kruskal-Wallis test: This test was applied to compare medians for ≥3 independent groups.

For calculation of NAR score, we applied the formula introduced by George et al., NAR=[5 pN- 3(cT - pT)+12]2 /9. 61 where cT(1, 2, 3, 4) , ypT(0, 1, 2, 3, 4), and ypN(0, 1, 2) [144].

NAR score is a pseudocontinuous variable with 24 discrete scores in the range between 0 and 100. The score is divided into three risk groups: low risk (<8), intermediary risk (8-16), and high-risk scores (>16) [144].

With ypT and ypN adopting the same range as above, a pathologic score could adopt a range 0-6 [145]. To differentiate between outcomes and categorization, we applied an arbitrary classification of low risk (0-2), intermediate risk (3-4), and high-risk score (5-6). In the same manner, a combined score (range 1-10) with incorporation of cT (1, 2, 3, 4) was divided into three risk groups, low (1-3), intermediary (4-6), and high score (7-10).

OS was calculated from time of surgery to death for any reason. Similarly, TTR (Time to recurrence of local/systemic failure or both) was calculated from time of surgery to any recurrence. Cancer specific survival (CSS), calculated from the same timepoint to death due to cancer disease. Reverse Kaplan-Meier was used to calculate follow-up.

4 RESULTS

4.1 STUDY I

The aim of this work was to identify substances with selective antitumoural activity against tumour cells with mutated p53.

In the first step, we performed a screen of the NCI library for natural products database with the aim of finding substances with inhibitory effects on tumour cell proliferation and selectivity for cells carrying mutant p53, and similar effects to PRIMA-1 which demonstrated such features [44].

Screening of substances with increasing concentration of N37063 in H1299 -/- (lacking p53) cells and comparison of effects in H1299-His175 (with mutation at residue 175) cells in the WST-1 proliferation assay demonstrated the selectivity of N37063 (Fig. 1A). Treating the H1299 cells with a dose of 2. 75 μg/ml N37063 toward mutant p53 resulted in only 38% cell survival while little effect could be detected in cells lacking p53 (Fig. 1). The same selectivity could be observed in Saos cell pairs (Fig. 1).

To check the effect of N37063 on cell cycle distribution, we performed analysis with PI-FACS. The mutant variants harbour mutant p53 constructs in which their expression could be switched off largely (but not completely) when pretreated with doxycycline (Fig. 2). In the next step, after pre-treatment with doxycycline and addition of test substance, less cell fragmentation and cell death were observed in PI-FACS indicating the need for functioning p53 pathway(s) for the effect of the extract (Fig. 2).

Next, we applied CaspaTag FACS to measure activation of the caspase system as a result of p53-dependent activation and cell apoptosis (Fig. 2). We confirmed a more pronounced activation of the caspase system in mutated cell lines compared to cells lacking p53 (Fig. 3A+3B). Again, the effect of test substance was diminished when cells were pretreated with doxycycline, confirming that N37063 exerts its effect mainly through the p53 pathway (Fig. 2).

Extending our analysis to other tumour types with wild type p53, we treated HCT116 cell lines (CRC) with N37063 (Fig. 3). The maximal inhibitory dose in HCT116+/+(wt) was 3. 1 μg/ml, while in HCT116 p53-/-(completely lacking p53) cells the maximal inhibitory dose was 5. 8 μg/ml, nearly double that of the wild-type variant, indicating the need for the p53 pathway for N37063-mediated cell death (Fig. 3).

As reactive oxygen species (ROS) are known to be an inducer of p53 activity [146], we hypothesized that some of the activity of N37063 could be related to activation of ROS, which in turn activate p53. As N-acetylcysteine (NAC) inhibits ROS activation, theoretically pre-treatment with NAC should result in decreased ROS and, consequently, diminished effect of N37063.

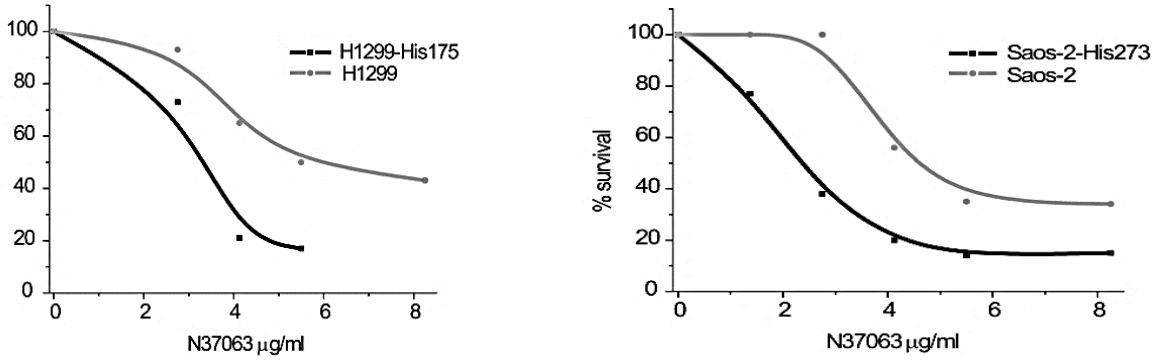

Fig. 1. N37063 induction of cell apoptosis in a p53-dependent manner in human tumour cell lines. **A:** Inhibition of cell growth in mutated p53 cell lines H1299-His 175 (left panel) and Saos-II His 273 (left panel).

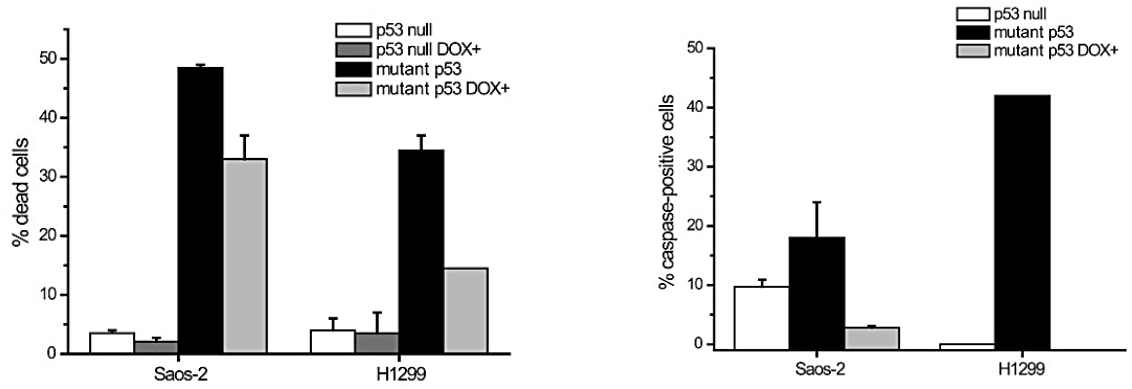

Fig. 2. DNA fragmentation indicating apoptosis measured by PI-FACS. Left: Quantitative demonstration of increased cell death in mutated Saos-II and H1299 cells but minimal effect on cell death in cells lacking p53. The apoptotic effect from the extract was reduced when pre-treated with doxycycline (to switch off expression of p53). Right: Treatment with N37063 induced more caspase-activated cells in mutated H1299-His 175 and Saos-II His 273 as measured by CaspaTaq-FACS. Dox, doxycycline; PI-FACS, propidium iodide fluorescence-activated cell sorting.

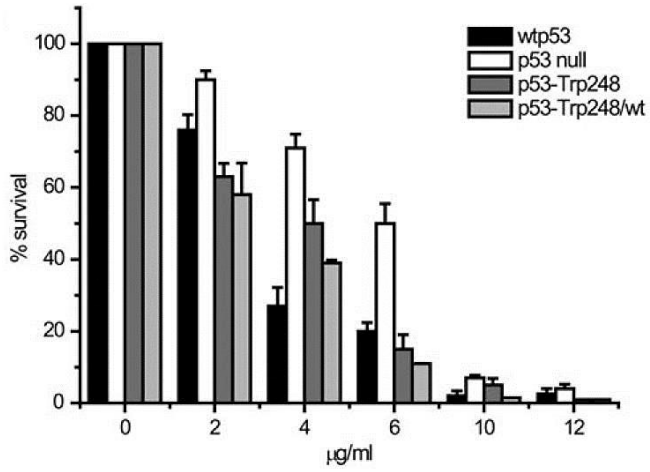

Fig. 3. WST-1 assay measuring cell growth inhibition after treatment with N37063 in CRC cell lines with different p53 gene status.

Lastly, we attempted to characterize and purify the active component(s) in the extract. As a screen, we performed hexane fractionation and the WST-1 assay was repeated for all obtained fractions on the H1299 cell line. Two substances were purified, kairatenyl palmitate and hopenyl palmitate, and were tested in the WST-1 assay for measuring cell proliferation inhibition (Fig. 7B). Both purified compounds demonstrated growth inhibitory effects in H1299 cell pair with selectivity for mutant p53 (Fig. 4).

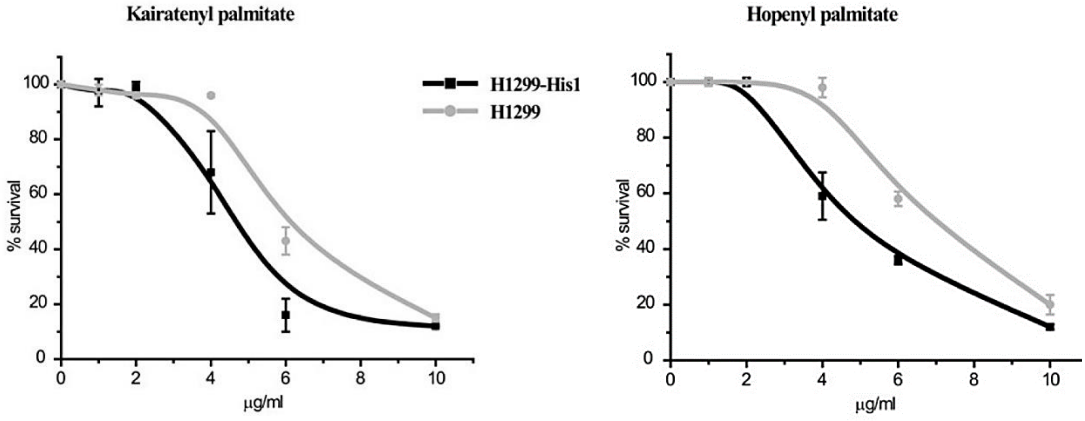

Fig. 4. Characterization of the active components in N37063. Both kairatenyl and hopenyl palmitate demonstrated mutant p53-dependent growth suppression in a p53-dependent manner in H1299-His175 cells as demonstrated by the WST-1 assay.

4.2 STUDY II

In total, we were provided with pedigrees for 235 LS families with verified LS mutations from five participating centres. A total of 1054 family members had at least one cancer diagnosis in their medical history with 445 cases (42%) cases with verified mutations, 343 cases (33%) who were obligate mutation carriers, and 265 (25%) cases assigned a 50% risk for being carriers. In total, 1495 cancer diagnoses could be verified in the study population. First-time CRC was registered for 647 individuals (43%) of which 148 patients experienced a second metachronous CRC (23%). EC was the second most

common cancer diagnosis in the LS cohort, responsible for 14% of all reported cancer cases (n=216). For men, CRC comprised 64% of all cancer cases, while in women the corresponding value was 36% followed by EC, 28%.

In total, 482 cases of non-CRC, non-EC could be found. To calculate relative proportions, we first excluded CRC and EC.

For the whole cohort, independent of MMR mutation type, a significantly elevated proportion, relative to the general population, was observed for non-prostatic urinary tract cancer, non-melanoma skin cancer, ovarian cancer, gastric cancer, and small bowel cancer (Table 4).

Next, we stratified the study cohort for gender and mutated MMR gene for subgroup analyses. In this analysis, both sexes in the LS group demonstrated a higher incidence of both gastric and small bowel cancer. Furthermore, women with LS experienced more ovarian cancer than the non-LS population.

Table 4. Observed cancer rates for the **Lynch syndrome cohort** with 100% or 50% probability of *MMR* mutation (after excluding CRC and EC). The observed proportions adjusted for sex and age are compared to those of the general population I (ref National Board of Health and Welfare)

	No of cases	Proportion in Lynch Cohort (%) (Lower limit-upper limit)	Proportion (%) in general population in Sweden 1970	Proportion (%) in general population in Sweden 2010	Proportion in Lynch in relation to general population
Urinary tract cancer #	75	15. 6 (12. 5-19. 0)	8. 9	5. 8	*above*
Gastric cancer	67	13. 9 (10. 9-17. 0)	6. 5	1. 4	*above*
Breast cancer	60	12. 5 (9. 6-16. 0)	17. 2	23. 8	*below*
Cancer of ovary and fallopian tube	43	9 (6. 5-11. 6)	5. 1	2. 1	*above*
Prostate cancer	38	7. 9 (5. 6-10. 4)	5. 0	12. 1	Within reference
Non-melanoma skin cancer	37	7. 7 (5. 4-10. 2)	1. 9	4. 8	*above*
Cancer of brain and nervous system	31	6. 4 (4. 4-8. 7)	6. 1	5. 0	Within reference
Haematological malignancy	20	4. 2 (2. 5-6. 0)	9. 8	9. 3	*below*
Pancreatic cancer	16	3. 3 (1. 9-5. 0)	2. 8	1. 7	Within reference
Cancer of small bowel	15	3. 1 (1. 7-4. 8)	0. 5	0. 6	*above*
Cancer of liver and biliary system	13	2. 7 (1. 5-4. 2)	2. 7	1. 7	Within reference
Malignant melanoma	13	2. 7 (1. 0-4. 6)	4. 5	9. 4	*below*
Cancer of lungs and major airways	10	2. 1 (0. 9-3. 5)	6. 9	5. 8	*below*

After stratifying for MMR gene mutation, we observed a higher proportion of gastric and small bowel cancers in the MLH1 group while non-prostatic urinary tract cancer was the most common malignancy in the MSH2 cohort, followed by cancer of the ovary in females with an MSH2 mutation (Tables 5 and 6). Of note was the finding that gastric cancer in the whole cohort was only 10% of patients with a diagnosis born after 1940 which could point to changing phenotype for LS depending on changing lifestyle, diet, and environment. A finding of an increased proportion of non-melanoma skin cancer seems to be mainly related to female patients with an MSH2 mutation.

4.3 STUDY III

The aim of this study was to explore whether routinely used pre-treatment clinical and laboratory parameters and MRI-defined staging features could predict a pCR state in pre-operatively treated RC patients. For this purpose, we retrospectively examined two independent cohorts, Uppsala-Dalarna (n=359) and Stockholm (n=635), who received pre-operative oncologic treatments scRT, CRT, or scRT/CRT+CTX and delayed surgery with curative intent. Additionally, for predictive factors that were identified, we constructed a predictive model for pCR state.

Minor differences in some clinical and imaging characteristics could be observed between the two cohorts such as age, cMRF+, cN+ and cEMVI+ and level of carcinoembryonic antigen (CEA). Due to different inclusion time, for Uppsala-Dalarna 2010-2018 and for Stockholm cohort 2006-2016, the proportion varied between treatment groups with more TNT in Uppsala-Dalarna and higher proportion of patients treated with CRT in Stockholm cohort. Different treatment schedules resulted in varied times to surgery.

The treatment groups varied in some characteristics as patients in the scRT cohort were older and had intermediary tumour features on MRI such as cMRF-, cEMVI-, and cN0. In contrast, the scRT/CRT group consisted mainly of patients treated in clinical trials who had more advanced tumours and were younger in age. The rate for pCR was nearly the same in both independent cohorts, 12. 8% in the Uppsala-Dalarna group and 12. 3% for the Stockholm cohort (Table 7). Median follow-up calculated for the pooled cohort was 64 months (95% CI 63-65). Recurrence-free survival (RFS) was significantly higher in the pCR group with 5-year RFS rates of 96% in pCR versus 79% in non-pCR groups. A 5-year OS rate of 92% was observed in the pCR cohort as compared with 70% in the non-pCR cohort.

Table 5. Comparison of clinical, imaging, and laboratory characteristics between pCR vs non-pCR groups

		Non-pCR n=870 (Row %)	**pCR n=124 (Row %)**	**P-value**
Age	Median (range)	68 (23-91)	65 (38-84)	***0. 003***
	≤ 70 years	531 (85%)	95 (15%)	***0. 001***
	> 70 years	339 (92%)	29 (8%)	
Sex	Female	351 (86%)	57 (14%)	0. 234
	Male	519 (89%)	67 (11%)	
MRI T-stage	cT1-2	48 (77%)	14 (23%)	***0. 027***
	cT3	464 (87%)	68 (13%)	
	cT4	357 (90%)	42 (10%)	
	Missing	1	0	
MRI N-stage	cN0	152 (89%)	19 (11%)	0. 546
	cN1-2	716 (87%)	105 (13%)	
	Missing	2	0	
MRI Tumour length	≤3. 5 cm	109 (80%)	27 (20%)	***0. 010***
	>3. 5 cm	716 (88%)	96 (12%)	
	Missing	44	1	
Weeks from end of RT	≤8	371 (90%)	43 (10%)	0. 110
to surgery	8-11	214 (88%)	29 (12%)	
	>11	285 (85%)	52 (15%)	
Haemoglobin	>110 g/L	689 (86%)	108 (14%)	0. 088
	≤110 g/L	97 (92%)	8 (8%)	
	Missing	84	8	
Leukocytes	≤10 ^9/L	614 (86%)	100 (14%)	***0. 014***
	>10 ^9/L	131 (94%)	9 (6%)	
	Missing	125	15	
Thrombocytes	≤400 ^9/L	418 (86%)	66 (14%)	***0. 023***
	>400 ^9/L	58 (97%)	2 (2%)	
	Missing	394	56	
C-reactive protein	≤10 mg/L	468 (86%)	77 (14%)	***<0. 001***
	>10 mg/L	160 (90%)	18 (10%)	
	Missing	242	29	
Carcinoembryonic antigen	≤5 µ/L	424 (84%)	80 (16%)	***0. 001***
	>5 µ/L	281 (92%)	26 (8%)	
	Missing	165	18	
Treatment group	scRT	402 (92%)	33 (8%)	***<0. 001***
	CRT	310 (87%)	48 (13%)	
	scRT/CRT + CTX	158 (79%)	43 (21%)	

Abbreviations: CRT: *concomitant chemoradiotherapy*, **MRI:** *magnetic resonance imaging*, **pCR:** *pathologic complete response*, **RT:** *radiotherapy*, **scRT:** *short course radiotherapy*, **scRT/CRT+CTX:** *scRT/CRT combined with systemic chemotherapy,* **MRI Tumour length***: craniocaudal extension of tumour measured by MRI. P-values below 0. 05 are marked in bold.*

The total pCR rate in the pooled cohort was 12% of the 994 patients. pCR was observed in 8% with scRT, 13% in CRT, and in 21% in scRT/CRT+CTX group (P<0. 001).

Comparison of the pCR vs non-pCR groups demonstrated tumour features, such as tumour length <3. 5 cm (p=0. 010) and MRI-defined cT-stage (p=0. 027), that were statistically associated with pCR. Furthermore, baseline laboratory parameters, such as elevated leucocytes (p=0. 014), thrombocytes (p=0. 023), CEA (p=0. 001), and increased CRP (p<0. 001) were significantly different between the two groups (Table 5).

Univariate binary logistic regression analyses for pCR revealed significant associations for age ≤70 years (OR 2. 09), tumour length ≤3. 5 cm (OR 1. 84), cT1-2 (OR 2. 47), time from end of RT to surgery (OR 1. 57), normal CEA (OR 2. 03), normal leukocytes (OR 2. 37), normal thrombocytes (OR 4. 57), or CRT (OR 1. 89) and scRT/CRT+CTX (OR 3. 32) (Table 6).

Table 6. Univariate and multivariate analyses of the pooled cohorts (n=994 and 735, respectively) for clinical, laboratory, and imaging-defined variables predicting pCR status.

		Univariate analyses n=994		Multivariable model n=735	
		OR (95% CI)	P-value	OR (95% CI)	P-value
Age	Continuous	0. 97 (0. 95-0. 99)	***0. 002***		
	> 70 years	1. 00		1. 00	
	≤ 70 years	2. 09 (1. 35-3. 23)	***0. 001***	1. 35 (0. 77-2. 37)	0. 291
MRI T-stage	cT4	1. 00		1. 00	
	cT3	1. 24 (0. 82-1. 87)	0. 292	1. 38 (0. 85-2. 28)	0. 193
	cT1-2	2. 47 (1. 26-4. 87)	***0. 008***	3. 37 (1. 30-8. 78)	***0. 013***
MRI tumour length	>3. 5 cm	1. 00		1. 00	
	≤3. 5 cm	1. 84 (1. 15-2. 96)	***0. 011***	2. 27 (1. 24-4. 18)	***0. 008***
Weeks from RT to Surg.	≤8	1. 00		1. 00	
	8-11	1. 16 (0. 70-1. 92)	0. 540	1. 61 (0. 87-2. 98)	0. 131
	>11	1. 57 (1. 02-2. 42)	***0. 040***	1. 45 (0. 79-2. 67)	0. 227
Leukocytes	>10 ^9/L	1. 00		1. 00	
	≤10 ^9/L	2. 37 (1. 16-4. 81)	***0. 017***	2. 02 (0. 93-4. 37)	0. 075
Thrombocytes	>400 ^9/L	1. 00			
	≤400 ^9/L	4. 57 (1. 09-19. 2)	***0. 037***		
Carcinoembryonic antigen	>5 μ/L	1. 00		1. 00	
	≤5 μ/L	2. 03 (1. 27-3. 25)	***0. 003***	1. 73 (1. 04-2. 90)	***0. 034***
Treatment group	scRT	1. 00		1. 00	
	CRT	1. 89 (1. 18-3. 01)	***0. 008***	2. 621 (1. 34-5. 14)	***0. 005***
	scRT/CRT + CTX	3. 32 (2. 03-5. 41)	***<0. 001***	4. 70 (2. 23-9. 93)	***<0. 001***

Abbreviations: CRT: *concomitant chemoradiotherapy*, **MRI:** *magnetic resonance imaging*, **pCR:** *pathologic complete response*, **RT:** *radiotherapy*, **scRT:** *short course radiotherapy*, **scRT/CRT+CTX:** *scRT/CRT combined with systemic chemotherapy,* **MRI tumour length***: craniocaudal extension of tumour measured by MRI. P-values below 0. 05 are marked in bold.*

For multivariate analysis, we included the covariates which showed significance in the univariate analyses. cT1-2 tumours (OR 3. 37), tumour length ≤3. 5 cm (OR 2. 27), normal CEA (OR 1. 73), and CRT (OR 2. 61) or scRT/CRT+CTX (OR 4. 70) remained significant as parameters associated with pCR in the pooled cohort, independent of treatment schedule (Table 6). Interaction analysis did not reach significance levels for the variables significant in multivariate analysis were not significant and thus not included in the multivariate model.

Furthermore, we examined association of predictive factors with pCR for the three treatment groups separately. In the scRT group, univariate analyses demonstrated significance for age ≤70 years, cT1-2 stage, cEMVI-, cMRF-, and thrombocytes within reference interval were associated with higher pCR rates but none of the factors remained significant in the multivariate analysis. In the CRT population, again cT1-2, tumour length ≤3. 5 cm, and normal CEA were associated with a higher pCR probability in the univariate analyses, with only cT1-2 (OR 5. 94) remaining significant in the multivariate analysis. In the scRT/CRT+CTX group, female sex with OR 2. 00 was the only significant factor in the univariate analyses.

Based on significant variables identified in multivariate analysis, we develop a predictive model for pCR and calculated AUC for model fitness (Table 7).

AUC as a measure for performance of the predictive model was 0. 65 for the pooled cohort and exhibited the best predictivity for scRT/CRT+CTX with 50% accuracy when the cut-off for scoring points was <1. 75.

Table 7. Score board for the predictive pCR model

			Non-pCR		pCR		Univariate analysis		Multivariate analysis	
		Points	n=869	Row %	n=124	Row %	n=811–993	*P*-value	n=735	*P*- value
Clinical T-stage	cT1-2	0. 0	48	77%	14	23%	2. 48 (1. 26–4. 87)	***0. 008***	1. 63 (0. 67–3. 95)	0. 278
	cT3	0. 5	464	87%	68	13%	1. 25 (0. 83–1. 87)	0. 292	1. 09 (0. 67–1. 77)	0. 723
	cT4	1. 0	357	90%	42	11%	1. 00		1. 00	
MRI tumour length	≤3. 5 cm	0. 0	109	80%	27	20%	3. 09 (1. 46–6. 50)	***0. 003***	3. 15 (1. 26–7. 86)	0. 114
	4–7 cm	0. 5	579	87%	85	13%	1. 83 (0. 95–3. 52)	0. 071	1. 87 (0. 86–4. 08)	0. 115
	> 7 cm	1. 0	137	93%	11	7%	1. 00		1. 00	
Leucocytosis	≤8 ^9/L	0. 0	458	85%	82	15%	2. 61 (1. 27–5. 23)	***0. 009***	2. 28 (1. 06–4. 94)	***0. 036***
	8–10 ^9/L	0. 5	156	90%	18	10%	1. 68 (0. 73–3. 86)	0. 223	1. 51 (0. 58–2. 26)	0. 367
	>10 ^9/L	1. 0	131	94%	9	6%	1. 00		1. 00	
Carcinoembryonic	≤3 µ/L	0. 0	289	82%	64	18%	2. 39 (1. 47–3. 89)	***<0. 001***	1. 85 (1. 10–3. 12)	***0. 020***
antigen	3–5 µ/L	0. 5	135	89%	16	11%	1. 28 (0. 66–2. 47)	0. 459	1. 15 (0. 58–2. 26)	
	>5 µ/L	1. 0	281	92%	26	9%	1. 00		1. 00	

Abbreviations: pCR: *Pathologic complete response*, **MRI tumour length***: craniocaudal extension of tumour measured by MRI. P-values below 0. 05 are marked in bold.*

4.4 STUDY IV

The aim of this study was to examine significant correlations between NAR score and its risk categories for three oncological outcomes, time-to-tumour recurrence (TTR), cancer-specific survival (CSS), and OS for all patients and for every preoperative treatment group separately. After including patients who met study-defined criteria with available medical records, NAR score was calculated for 1009 patients. Additionally, we evaluated whether the predictive power of the NAR score could be improved by integrating other imaging or histopathologic prognostic parameters. The prognostic power of the NAR score was compared with the pathological score ypT+ypN for predicting different outcomes.

Relapse and death

A total of 266 (26%) patients experienced disease recurrence, of which local relapse was noted in 54 (5. 3%) and 250 (25%) experienced distant metastases. Of these 266 relapses, 230 (86%) occurred within 3 years. There were 174 (17%) cancer deaths and 148 (15%) non-cancer related deaths during follow-up.

In univariate analyses, cEMVI was the only pre-treatment parameter significantly associated with TTR with a C-index of 0. 547. Most pathologic factors, ypT, ypN, ypCRM, ypEMVI, and ypPn, were significantly associated with TTR, with c-indexes in the range of 0. 528-0. 671.

Median NAR score in the whole group was 14. 9 (range 0-65), being 14. 9 (IQR 8. 4-23. 4) for scRT, 12. 6 (8. 4-20. 4) for CRT, and 8. 4 (8. 4-20. 3) for scRT/CRT+CTX (p=0. 001, Table 8). The CRT and scRT/CRT+CTX groups had higher rates of low-NAR patients than the scRT group, and the scRT/CRT+CTX group had a lower rate of NAR-high patients than the CRT or scRT groups.

In all patients, TTR, CSS, and OS differed significantly between the low-, intermediate-, and high-NAR groups (Fig. 5 C, F, I). However, the strongest associations were found for TTR (HRs for the high-risk score 11. 2 for TTR, 9. 6 for CSS, and 4. 3 for OS) (Fig. 8). This was also true for all three treatments separately regarding TTR, but for CSS and OS it was not always statistically significant for the intermediate group in the CRT and scRT/CRT+CTX groups.

Five-year TTR rates for treatments were 92%-98% in the low-score group, 73%-85% in the intermediate group, and 46%-58% in the high-risk group.

The C-indexes for NAR were calculated both as continuous or categorical variables and varied between 0. 621-0. 716 for all patients, with the highest values noted for TTR. When calculating C-index for the three treatment groups separately, the highest value was found in the scRT/CRT+CTX arm and the lowest for the scRT group for all outcome variables measured, TTR, CSS, or OS. Measuring the prognostic correlation with outcomes with AIC instead of C-index confirmed the same tendency with the best correlation for scRT/CRT+CTX.

We examined whether the NAR score had more prognostic information for the outcomes of interest for the whole cohort and the three treatment schedules, respectively,

than the pathologic and combined models consisting of cT, pT, and pN (constituting the individual parameters in the NAR formula but without weighing them in mathematical terms as in the NAR formula). Both the pathologic and the combined scores correlated significantly with outcomes, TTR, CSS, and OS (Table 12).

When comparing pathologic model, combined score, and NAR score, the highest C-index values were noted for the pathologic model (0. 718 for TTR, 0. 726 for CSS, and 0. 659 for OS). The NAR groups had the highest C-index for TTR (0. 716), and the pathologic score for CSS (0. 721) and OS (0. 652). The AIC had the lowest and, thus, the best values for the pathologic score, compared with combined and NAR scores (Table 12).

The NAR score model for TTR in the whole group was combined with other significant prognostic parameters in the univariate analyses: ypEMVI (stronger than cEMVI which was not included due to significant interaction term), ypPn, ypCRM, and ypMucin (Table 2) in a multivariate forward model (shown in the lower part of Table 4). The NAR+ypEMVI+ypPn model had the strongest prognostic information for the whole group, with C-index 0. 737 for TTR, 0. 744 for CSS, and 0. 661 for OS (Table 12).

Of note, we found that the pathologic, combined, NAR, and NAR+ypEMVI+ypPn models varied in predictive strength between the different treatments with the lowest C-indexes for scRT (0. 625-0. 690), for CRT (0. 645-0. 729) and the strongest model fitness for scRT/CRT+CTX (0. 720-0. 791) concerning TTR (Table 5), and the same trends were seen for CSS and OS. For TTR, the highest C-indexes were seen for NAR+ypEMVI+ypPn in all three treatment arms with C-index 0. 690-0. 791. AIC values are generally in line with C-indexes but with smaller differences as there are three variables in the NAR+ypEMVI+ypPn model.

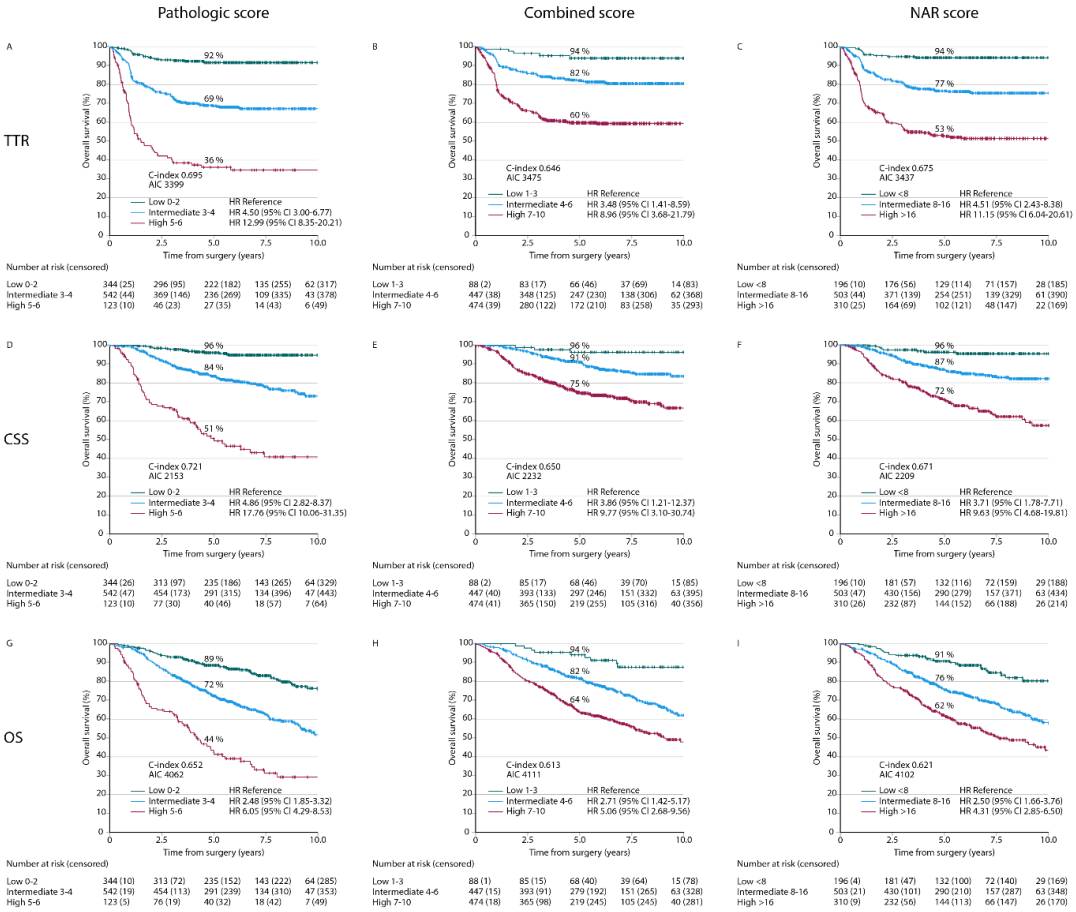

Figure 5. Time-to-recurrence (TTR; A, B, C) cancer-specific survival (CSS; D, E, F), and overall survival (OS; G, H, I) by pathologic score (A, D, G), combined score (B, E, H), and NAR score (C, F, I) low, intermediate, and high. AIC can only be compared horizontally

Abbreviation: ***AIC***: Akaike Information Criterion, ***CI:*** confidence interval, ***Combined score:*** cT+ypT+ypN***, CRT***: Concomitant radiochemotherapy, ***EMVI***: Extramural vascular invasion, ***HR:*** hazard ratio, ***NAR***: Neoadjuvant Rectal Score***, Pathological score:*** pT+pN ***pCR***: Pathological complete regression, ***scRT***: short course radiotherapy, ***scRT/CRT+CTX***: scRT or CRT preceded or followed by systemic chemotherapy. Best C-index and AIC in bold.

Table 8. Cox regression analyses, C-index, and Akaike information criterion (AIC) of time-to-recurrence (TTR), cancer-specific survival (CSS), and overall survival (OS) for pathologic, combined, NAR, and NAR with pathological variables scores* for all patients:

		TTR All patients n= 1007						**CSS All patients n=1009**						**OS All patients n= 1009**					
		P-value	HR	95% CI Lower	95% CI Upper	C-index	AIC	P-value	HR	95% CI Lower	95% CI Upper	C-index	AIC	P-value	HR	95% CI Lower	95% CI Upper	C-index	AIC
Pathological score	Continuous	***<0. 001***	1. 90	1. 72	2. 11	0. 718	**3383**	***<0. 001***	1. 99	1. 75	2. 26	0. 726	2157	***<0. 001***	1. 54	1. 41	1. 68	0. 659	**4061**
	Low (0–2)	***<0. 001***	1. 00					***<0. 001***	1. 00					***<0. 001***	1. 00				
	Intermediate (3–4)	***<0. 001***	4. 50	3. 00	6. 77	0. 695	3399	***<0. 001***	4. 86	2. 82	8. 37	0. 721	**2153**	***<0. 001***	2. 48	1. 85	3. 32	0. 652	4062
	High (5–6)	***<0. 001***	12. 99	8. 35	20. 2			***<0. 001***	17. 76	10. 06	31. 35			***<0. 001***	6. 05	4. 29	8. 53		
Combined score	Continuous	***<0. 001***	1. 63	1. 50	1. 78	0. 700	3413	***<0. 001***	1. 74	1. 56	1. 93	0. 720	2168	***<0. 001***	1. 44	1. 34	1. 55	0. 660	4062
	Low (1–3)		1. 00					***<0. 001***	1. 00					***<0. 001***	1. 00				
	Intermediate (4–6)	***<0. 001***	3. 33	1. 81	6. 14	0. 654	3466	***0. 023***	3. 86	1. 21	12. 37	0. 650	2232	***0. 002***	2. 71	1. 42	5. 17	0. 613	4111
	High (7–10)	***<0. 001***	7. 39	4. 12	13. 25			***<0. 001***	9. 77	3. 10	30. 74			***<0. 001***	5. 06	2. 68	9. 56		
NAR score	Continuous	***<0. 001***	1. 05	1. 04	1. 06	0. 700	3417	***<0. 001***	1. 05	1. 04	1. 06	0. 712	2180	***<0. 001***	1. 04	1. 03	1. 04	0. 642	4085
	Low (<8)		1. 00					***<0. 001***	1. 00					***<0. 001***	1. 00				
	Intermediate (8–16)	***<0. 001***	4. 51	2. 43	8. 38	0. 716	3437	***<0. 001***	3. 71	1. 78	7. 71	0. 671	2209	***<0. 001***	2. 50	1. 66	3. 76	0. 621	4102
	High (>16)	***<0. 001***	11. 15	6. 04	20. 61			***<0. 001***	9. 63	4. 68	19. 81			***<0. 001***	4. 31	2. 85	6. 50		
NAR + ypEMVI + ypPn *																			
NAR-score	Continuous	***<0. 001***	1. 04	1. 03	1. 05	**0. 737**	3387	***<0. 001***	1. 04	1. 03	1. 05	**0. 744**	2158	***<0. 001***	1. 03	1. 02	1. 04	**0. 661**	4073
Pathological EMVI	ypEMVI+	**0. 002**	0. 62	0. 46	0. 84			0. 727	1. 07	0. 73	1. 56			0. 831	0. 97	0. 72	1. 30		
Pathological	ypPn0	**0. 003**						***<0. 001***						***<0. 001***	1. 00				
perineural invasion	ypPn1	**0. 001**	1. 65	1. 23	2. 22			***<0. 001***	2. 23	1. 55	3. 21			***<0. 001***	1. 67	1. 26	2. 22		
	ypPn missing	0. 270	1. 75	0. 65	4. 72			***0. 017***	3. 41	1. 25	9. 29			0. 031	2. 29	1. 08	4. 88		

*Not included in the forward model: pathologic complete response, pathologic circumferential margin, and pathologic mucinous tumour

Abbreviation: ***AIC***: Akaike Information Criterion, ***CI:*** confidence interval, ***Combined score:*** cT+ypT+ypN***, CRT***: Concomitant radiochemotherapy, ***EMVI***: Extramural vascular invasion, ***HR:*** hazard ration, ***NAR***: Neoadjuvant Rectal Score***, Pathological score:*** pT+pN ***pCR***: Pathological complete regression, ***scRT***: short course radiotherapy, ***scRT/CRT+CTX***: scRT or CRT preceded or followed by systemic chemotherapy. Best C-index and AIC in bold.

5 DISCUSSION

Study I addressed obstacles to treatment in p53-mutated cancers which have a tendency for more aggressive behaviour and higher tumour resistance to available chemotherapy [37, 38]. By screening the NCI dataset for natural products, we found a substance that demonstrated, in vitro, a preferential effect on tumour cells with mutated p53. We also noted that an extract from the plant *B. ramiflora* exhibits antiproliferative and cytotoxic effects against cell lines from CRC, lung cancer, and sarcoma cell lines. After purification, this preferential cytotoxic effect on mutated p53 could be traced to two organic substances, kairatenyl palmitate and hopenyl palmitate.

P53 has been associated with two contrasting effects in cancer treatment. Many cytostatic and RT treatments rely on intact wild type p53 activation to mediate cell arrest and cell death. On the other hand, mutation of p53 promotes cancer progression, tumourigenesis, and drug resistance [28, 147]. Theoretically, p53 can be targeted in two major ways: (1) Enhancing wild-type p53 activation or more effective signaling in cells with wild-type p53 to induce outcomes such as cell death in response to chemotherapy or (2) targeting mutated p53 through inhibition or degradation. The first approach has generated more enthusiasm and is presumably more practical as several strategies can be applied such as selective inhibition of MDM2, which is responsible for negative feedback and degradation of p53 to low levels. This strategy has led to development of several inhibitory small molecules. Nutilin-3a is a small molecule inhibitor of the interaction between MDM2 and p53 that results in p53 stabilization and activation [148]. Many such compounds that utilize the same mechanism for stabilization of wild-type p53 are in phase I/II clinical trials, as single or combination therapy with immunotherapy against CRC or other solid tumours [149]. A critical aspect here will be tumours with mixed wild-type and mutated p53 as this can lead to stabilization of mutated p53 as well as wild-type p53 with deleterious effects on outcomes, requiring stratification for p53 intratumourally.

The second approach is to target mutated p53 and this can be approached in several ways. As 50% of tumours harbour mutated p53, which accumulates in tumour cells while normal cells usually lack mutated p53, it makes this strategy attractive to spare normal cells with wild-type p53 and, at the same time, target tumour cells [150]. This could be achieved by restoration of mutated p53 activation, inhibition of downstream mechanisms promoting survival, and inhibition of protein-protein interactions.

Restoration of wild-type p53 function in mutated cells is an approach that targets p53 by restoration of its native conformation, thus enhancing the DNA binding capacity of p53 and its function as a transcription activator and activating target genes to induce cell cycle arrest [151]. Some animal studies have concluded that restoration of wild-type function in p53-mutated tumours results in tumour regression and could prolong survival [151]. Some compounds have been reported to induce a wild type p53 native configuration in a mutated protein [43]. PRIMA-1met is a drug with the putative effect of restoration of native configuration of mutated p53 by refolding and is undergoing a phase I/II trial in combination with pembrolizumab against solid tumours [152, 153].

HSP90 and histone deacetylase (HDAC) inhibitors utilize another mechanism to target mutated p53 by increasing its degradation. Recently, the FDA has approved HDAC inhibitors for clinical use as cancer therapy in solid tumours [154, 155].

In summary, p53 is a central hub in integrating diverse stress signals and translates these signals into an appropriate outcome. How this integration and translation occurs in a cell- and tissue-specific manner remains largely unknown. Most knowledge about p53 comes from cell and animal studies with limited translation of findings to a human tissue context. Organoids, cells that can organize themselves in tissue culture mimicking normal tissue with three-dimensional growth, can be applied to study p53 function in a more authentic situation than cell cultures [156].

Single-cell methodology is another method for analysis of whole genome, transcriptome, and protein expression at the single cell level. This experimental procedure can help to broaden our understanding of p53 function in a tissue-specific manner. Questions such as the higher observed p53 mutation rate in the left colon and rectum compared to the probarrowximal colon would be interesting to study with both single-cell and organoid methods.

The established knowledge regarding how p53 suppresses tumourigenesis is undergoing a radical change. Previously, cell cycle arrest and apoptosis were believed to be the main mechanisms of how p53 exerts its function as a tumour suppressor. This knowledge has been challenged recently as other mechanisms, such as a role in drug resistance, migration, invasion, metastatic potential and metabolism, are emerging [157]. Elucidating how p53 exerts tumour suppression could lead to new therapeutic option in cancer therapy.

In **Study II**, we investigated the spectrum of extracolonic, non-endometrial cancers in Swedish Lynch syndrome families under surveillance. We confirmed that predisposition for different cancer diagnoses varies according to which MMR gene is mutated. Our results show some slight variations in frequency and specific cancer risk for LS individuals in comparison to previous publications aiming to characterize tumour spectrum in LS [158-160].

Although CRC, EC, and urothelial cell cancers are an accepted part of the LS spectrum, there has been conflicting data about other diagnoses such as breast cancer, CNS tumours, lymphomas, and other cancers [158, 160-162]. CNS tumours have been suggested to be a part of LS in several studies [158, 163]. A British study confirmed an association between LS and CNS tumours [158]. Neither CNS tumours nor breast cancers were confirmed as part of LS in our cohort. Most of the published studies have been of a retrospective nature, using different methodologies to define frequency and proportion, and different population sizes. This has led to some conflicting results. As these studies have been conducted in other countries, one explanation for controversy regarding the LS spectrum could be related to different ethnicities, variations in genetic background, and phenotypic manifestations that are influenced by environment. The importance of phenotypic manifestations in different genetic backgrounds has been illustrated in a multinational cohort study of LS families in the US, the Netherlands, Denmark, and Finland. In this study, a significantly higher incidence of urothelial cell cancer was noted in a Danish and Finnish registry compared to LS [160] in the Netherlands and US.

The finding that gastric cancers in the older generation decreased in incidence over time in our LS cohort was also in line with a report from a British study [158]. According to the Swedish National Board of Health, the proportion of gastric cancer has been steadily decreasing between 1970 and 2010 in the general population. Our results show the same tendency in our Swedish LS cohort and suggest that the LS tumour spectrum may follow the spectrum in the general population. This finding could also point to variation in the cancer spectrum over time with changing environmental and genetic factors. As we demonstrated, the cancer spectrum in Lynch families varied over time and generations in response to ill-defined environmental factors and follows roughly the epidemiology of the cancer spectrum for the general population.

Another important finding was that Lynch syndrome and its predisposition for different malignancies varies substantially across different MMR gene mutations. Patients with MLH1 mutations are at increased risk for gastrointestinal malignancies while patients with an MSH2 mutation are more affected by urothelial cell cancers and non-melanoma skin malignancies. This implies that surveillance studies as a preventive measure need to be stratified according to MMR gene mutation as the risk varies for different cancer diagnoses.

In **Study III**, we examined a broad range of clinical, radiologic, and laboratory parameters for their ability to predict a pCR state after preoperative oncologic treatment. Achieving a pCR state confers excellent oncologic outcomes and the opportunity to be managed by non-operative measures such as a "watch and wait" approach. This has driven the effort to identify parameters that are predictive for pCR. Much of that interest has been focused on the time interval from the end of radiation to surgery and on CEA levels. Of 994 patients included in our study cohort, 78 patients (12. 3%) achieved a pCR, in line with previous results from other studies [101, 103, 105, 120, 121, 164], especially considering the high rate of LARC in our study cohort.

A comparison between the pCR vs non-pCR groups demonstrated that the groups differed significantly in terms of delivered neoadjuvant regimen, age, imaging features such as tumour length and MRI-defined T-stage, laboratory parameters such as CEA, leukocytes, and thrombocytes.

The preoperative treatment schedule delivered was the most decisive factor to achieve pCR with an OR of 4. 70 for TNT in multivariate analysis followed by CRT, OR 2. 621. The hierarchy of efficacy of preoperative treatment in attaining pCR with scRT/CRT+CTX as the best choice followed by CRT and least for scRT is in line with previous reports [165, 166].

The data need to mature regarding how TNT impacts DFS and OS. Some questions need to be answered before TNT is accepted completely, such as whether CRT is superior to scRT in the context of TNT (as was observed in RAPIDO), induction vs consolidation (OPRA with higher TME-free survival in the consolidation arm than the induction arm), doublet (CAPOX/FOLFOX) vs triplet FOLFIRINOX, duration of chemotherapy before surgery and, lastly, whether to possibly to omit RT from pre-operative treatment for less advanced rectal tumours.

In summary, the hierarchy we noted in our study for pCR achievement for the three common preoperative treatment schedules agrees with previous studies [130, 131, 134, 143, 167].

The higher pCR rate observed for TNT will likely make this treatment the first choice for RC with the aim for an organ-preserving approach. scRT with delay seems to not be the best option when considering organ preservation.

The finding of low CEA correlated with higher pCR rate was in concordance with previous studies that have demonstrated repeatedly that CEA is a predictive factor for pCR as an independent parameter in multivariate analysis [167-173]. There are, however, some reports indicating that the dynamics and clearance of CEA during preoperative treatment is the more important factor to predict pCR [174-176]. CEA is roughly correlated to tumour burden. A higher value of CEA has been observed in smokers than non-smokers. Nearly all studies investigating CEA as a predictor for pCR have been small retrospective studies with no stratification for smoking. Additionally, different cut-off values for CEA have been applied in different studies with positive results. Although easily available and analysed, its predictive power apparently is limited with inadequate sensitivity and predictive strength for pCR.

Blood chemistry with haemoglobin, leucocytes, and thrombocytes have also been in a focus of interest as pCR predictors [172, 173, 177-179]. In our results, leucocytes demonstrated significance when comparing pCR vs non-pCR and in univariate analysis for all patients.

As with CEA, previous publication used different cut-off values for blood chemistry. Furthermore, blood chemistry offers both categorization in statistical analysis as well as measuring as continuous variables which resulted in conflicting conclusions.

Ramsay examined the role of pre-treatment blood chemistry in 330 patients, including 71 patients with pCR, treated preoperatively, and reported leucocytes as the most important predictor for pCR in routine haematology tests [177]. The predictive power of leucocytes was observed with an AUC equal to 0. 666, a rather modest discriminating power for a predictor [177]. In publications from both Joye and Armstrong, a higher haemoglobin level was associated with higher probability for pCR, but this could not be reproduced in our results [172, 173]. Thrombocytes were not included in baseline blood chemistry in the Uppsala-Dalarna cohort but was a significant variable when comparing pCR vs non-pCR and univariate analysis in both scRT and for the whole cohort. The limited discriminative power of routine blood chemistry for pCR has led to more advanced analysis such as neutrophil to lymphocyte ratio with significance in small studies [179, 180]. Routine blood chemistry can demonstrate variations other than relation to cancer diagnosis and this could explain highly variable findings of these variables as predictive factors in previous studies.

The radiologic features of rectal tumours and associations with pCR have been the focus of much interest. MRI-defined cT stage and tumour length as predictors for pCR were studied previously [172, 181-185]. The impact of cT stage on achievement of pCR according to multivariate analysis was that for tumour length less than 3. 5 cm, the OR was smaller than for tumour length 2. 27 with the highest significance for CRT and to a lesser degree TNT. Joye published a predictive model which included low pre-treatment CEA and low cT stage as these were independent significant predictive parameters of pCR in that study. The calculated AUC reached only 0. 609 and the study concluded that the predictive power was so restricted that other strategies should be explored [172] despite the significance of the finding as an independent parameter in multivariate analysis.

Another study published by Lee reported an association between cT and pCR, but their conclusion was to use cT stage in combination with MRI-TRG to evaluate pCR state reached [183].

Our results point in the same direction as our predictive model's AUC for all patients reached 0. 65 which clearly demonstrate its limited predictive power to safely defer surgery in neoadjuvant-treated patients.

While there are many reports of cN0, cMRF-, and cEMVI- as predictors for pCR [170, 181, 185-187] , we could not reproduce the impact of these variables on pCR for the whole cohort but both factors demonstrated a negative impact on pCR in univariate analysis for scRT but not for CRT or TNT. One explanation could be that positive MRF and EMVI usually indicate a more advanced tumour. This, combined with a finding that fewer patients achieved a pCR in the scRT group, indicates that for a younger, fit patient, treatment with scRT for more advanced tumours is insufficient. Concerning N-state, the lower accuracy of MRI for identifying metastatic lymph nodes has been a diagnostic challenge with both overstaging and understaging [48, 56].

Based on findings of significant parameters in univariate and multivariate analysis, we constructed a predictive model based on cT, tumour length, CEA, and leucocytes. The predictive power of the model obtained an AUC of 0. 65 with cut-off of >1. 5 score for the whole cohort. The best discriminative power was seen for scRT/CRT+CTX when we applied a cut-off ≤1. 5 scoring points and with AUC 0. 65, 50% of patients achieved pCR.

The results demonstrated in Study III also have clinical implications. The predictive power of around 0. 6-0. 7 is not associated with a robust predictive tool but can be applied, in combination with other available tools, for selection of patients for an organ-preserving approach and avoiding permanent stoma in some selected cases. A T2/T3a 3-4 cm above the anal verge is treated with removal of the rectum without need for any preoperative oncologic treatment and permanent stoma, according to Swedish national guidelines for RC. In such cases, a younger fit patient can be offered neoadjuvant scRT/CRT+CTX if MRI defines the tumour as cT2/T3a/b, 2-3 cm in length and with normal CEA and leucocytes with a 50% probability for the patient to avoid more extensive surgery and permanent stoma. We believe that other methods with more robust predictive performance are needed to predict pCR in a patient safe manner.

In **Study IV**, we found a significant correlation for NAR score as a short-term surrogate endpoint for OS in RC patients treated preoperatively with any of three commonly used neoadjuvant treatment regimens, ScRT, CRT, or ScRT/CRT+CTX with delayed surgery. Previous studies established the significant correlation between OS and three NAR categories in retrospective studies mainly of CRT [188-191]. A Dutch retrospective registry study concluded that the already established ypT+ypN (the pathological score) performs better in correlation with OS after CRT and was superior to NAR score [145]. A Swedish nationwide registry study confirmed the results from the Dutch study and applied the conclusion to TNT and scRT too [192]. To our knowledge, no publication yet has studied NAR score for other oncologic outcomes such as TTR and CSS.

Contrary to previously published studies that examined NAR score significance for only OS, we also included TTR and CSS in addition to OS to investigate how NAR score

correlates with these outcomes, which are more cancer-specific than OS. In clinical oncology studies, a convention has been established to translate the efficacy of any drug/treatment schedule into OS which includes every death irrespective of the cause. Every death regardless of whether the cause is cancer, heart or hepatic failure in recurrence-free patients, or natural death. This way of calculating OS can be misleading, especially in the older patient category, as many have other comorbidities which are age related and the group ultimately dies as a result of non-malignant disease or age.

Our results demonstrate a strong correlation between NAR score and all three outcomes examined, TTR, CSS, and OS for all three pre-operative treatments with the best fitness for TTR followed by CSS and least for OS. This could be explained by our previous argument about TTR and CSS which are more relevant for RC patients. Another finding in our results was that the NAR model's performance varied according to preoperative treatment delivered with best performance for scRT/CRT+CTX and worst for scRT. One explanation for this finding could be that the scRT/CRT+CTX cohort in our study was comprised of much younger patients with generally more advanced tumours and was more homogeneous considering tumour features such as cT, N+, cMRF+ while the scRT group contained older patients with less advanced tumours and clearly more heterogenous tumour features. Another explanation of better fitness of NAR with scRT/CRT+CTX might be related to downstagings efficacy. NAR score contains a measurement of T migration from baseline imaging to ypT. The same pattern is noticeable for NAR score fitness for treatment schedule studied. A third explanation could be that NAR score exhibits its best performance with more advanced tumours which were mainly included in the scRT/CRT+CTX and CRT groups.

We compared NAR score strength for the chosen outcomes with the pathologic model (ypT+ypN) and the combined model (cT+ypT+ypN). The combined model is a theoretical model which cannot be translated into a score utilized in everyday practice. We confirmed the superiority of the pathologic model compared to NAR score for all three outcomes examined.

In our attempt to improve the prognostic power of the NAR, we analysed the addition of other radiological and pathological parameters which could have impact on patient outcomes. The only imaging parameter which demonstrated significant correlation with TTR was cEMVI+ while all pathological variables (except for tumour deposit) were associated significantly with TTR. We chose the two variables pEMVI and ypN (perineural invasion on pathological specimen) with the best C-index and recalculated for the outcomes and compared with the pathological and combined models. This yielded a superior fitness for NAR+ypEMVI+ypPn compared to both the pathological and combined models for the whole cohort and for TTR and CSS for treatment groups, indicating potential for modification and improvement of the NAR score. We concluded that NAR prognostic strength shows marginal room for improvement. We are not aware of any previous publication examining improvement of NAR score prognostic strength by incorporation of other prognostic parameters.

Efforts have been initiated to optimize and improve NAR score performance with introduction of other risk groupings than those proposed initially by George [20, 37, 57]. An abstract was presented with regrouping of high-risk score into two groups, 16-26. 6 and >26. 6 resulting in 4 risk categories and better performance for survival. Another way to

improve NAR performance is subgrouping into T3 categories (T3a, b, c, d). In such a situation, a downstaging from T3c/d to T2 can be assigned a different score than downstaging from T3a to T2 creating new risk groupings [193].

Other short-term surrogate has been proposed as a measure for efficacy of preoperative treatments such as pCR vs non-pCR with established significant correlation to OS [143]. pCR vs non-pCR is a two-tier system with lower performance in the non-pCR group while NAR score is a three-tier risk grouping with higher discrimination in the same group of patients. Comparison between pCR vs NAR score and their correlation to OS was discussed in the form of an abstract which showed nonsignificant better predictive power for NAR than pCR in predicting OS [188].

NAR score is easily reproducible, rapidly calculated, and requires no extra procedure to obtain and can be available at post-operative multidisciplinary team discussion. One disadvantage could be that it relies heavily on precise pre-operative imaging staging which demands highly experienced radiologists and expertise.

George and Yothers, who invented and introduced the NAR score, clearly stated the role of NAR score as a short-term surrogate to measure efficacy of preoperative treatment and a more rapid integration of more efficient treatments in late clinical trials and not primarily as an individual prognostic tool [144]. Our results confirm the same conclusion as we noted better performance of the pathologic model for predicting OS compared to the NAR score.

Pathological TNM staging, albeit the strongest prognostic instrument in hand for predicting outcome, is not informative about the dynamic process that is initiated from the start of neoadjuvant oncologic treatment, with variable tumour response until surgery. Histopathologic TNM doesn't take into consideration tumour biology and responsiveness to available oncologic treatments. The prognosis for all ypT3a/b N1 is the same regardless of pre-treatment starting point, whether it was a T4bN2 or T2N0 tumour. NAR score reflects, to a limited degree, this dynamic process taking treatment response to neoadjuvant treatment into consideration and incorporating cT stage into its formula, providing us with information about what occurred from the time of initiating preoperative treatment until surgery. NAR score is not completely indifferent to the stage migration and tumour downstaging that occur during neoadjuvant treatment of RC patients, in contrast to histopathologic TNM staging. According to NAR, a T4 N2 RC that is downstage to T3N0 is classified as low-risk group compared to a T3N0 tumour with no response to ScRT that is stratified to the intermediary risk group.

Our knowledge about tumour response to oncologic treatments, regardless of intention, is still limited. Efforts to elucidate the inherent tumour features and molecular profiles underpinning responsivity to RT and CTX have not yet resulted in any consistent findings applicable in clinical practice (exceptions exist, such as EGFR treatment in *KRAS* and *BRAF* wild type tumours). But it is still clinical "common sense" that good responders have better prognosis than those with a non-responding tumour. NAR provides us with such a, albeit rough, numeric probability for this responsivity.

In summary, we believe pathological TNM is still the best tool at hand for predicting recurrence and OS. Although NAR exhibited significant correlations with outcomes, its utilization should be restricted to measurement of preoperative treatment efficacy and

translating this efficacy into a single value. NAR needs further optimization before it can be applied as an individual prognostic tool.

6 FUTURE DIRECTIONS

An era characterized by introducing targeted therapy and immunotherapy has evolved in clinical oncology since a few years. To find in advance molecular, imaging, or other clinical parameters predicting response to preoperative treatment would affect clinical decision-making avoiding treatment with significant toxicity to those unlikely to have benefit from treatments and at the same time offer preoperative treatment to others with the aim of organ preserving approach.

The strength of the predictive pCR model will be subject to validation in a cohort consisting of patients included in the RAPIDO phase III trial (n=920). This could confirm the strength and limitation of the pCR model in patients treated in a prospective randomized study.

Still, regarding the limited predictive strength in our study and similar publications so far, based only on incorporation of basic clinical and imaging variables, other innovative measures are needed to improve performances of the predictive models.

MRI radiomics is an expanding field based on extraction of high-throughput imaging characteristics that cannot be recognized by visual inspection but with the help of algorithms developed. There has been on use of radiomics in rather large sample size studies demonstrating a predictive performance with AUC above 0. 8 [194].

Another growing field of research with great potential is the role of artificial intelligence in imaging. Several small size studies have been already published but a large Chinese meta-analysis of 21 publications with 1562 patients in a training cohort and 1872 in a validation cohort of MRI-based artificial intelligence models exhibited a pooled AUC of 0. 91 (pooled sensitivity 82%, pooled specificity 86%) [195].

Our results showed some marginals for improving prognostic strength of NAR score for oncologic outcomes. As with pCR predictive model, NAR+ypEMVI+ypPn will be subject to validation in the RAPIDO material. We plan to examine NAR improvement by applying difference score, a change of prognostic parameters from baseline imaging to histopathology and incorporation with the NAR model.

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