UNIVERSITÀ DEGLI STUDI

DI MODENA E REGGIO EMILIA

Dottorato di ricerca in

Clinical and experimental medicine (CEM) - Medicina clinica e sperimentale

Ciclo XXXIII

Acute Radiation Colitis after Preoperative Short-Course Radiotherapy for Rectal Cancer: A Morphological, Immunohistochemical and Genetic Study

Candidato: Dott. MAURIZIO ZIZZO

Relatore (Tutor): Prof. FILIPPO LOCOCO

Coordinatore del Corso di Dottorato: Prof. GIUSEPPE BIAGINI

INDEX

1. Abstract

(ENGLISH)

Background: Preoperative radiotherapy with or without chemotherapy has been demonstrated of value in reducing local recurrence rates and improving overall survival in rectal cancer. Radiation-induced changes in the tumour are well described, whereas less attention has been given to the non-neoplastic mucosa. Our aim is to provide a detailed analysis of morphological, immunohistochemical and genetic features present in non-neoplastic mucosa. Pathologists need to be familiar with aforementioned morphological features, when evaluating rectal cancer specimens of patients preoperatively treated with radiotherapy, especially with short-course regimen, in order to avoid misdiagnosis.

Methods and Results: We compared 2 groups of 95 rectal cancer patients treated preoperatively with either short-course (25 Gy administered in 5 consecutive days, followed by surgery a few days after; 45 patients) or long-course radiotherapy (45-50 Gy in 4-6 weeks, followed by surgery 4 weeks later; 50 patients). Depending on the type of protocol, different histopathological features, in terms of inflammation, glandular abnormalities and endocrine differentiation were seen in the non-neoplastic mucosa within the irradiated volume. Of note, features mimicking dysplasia, such as crypt distortion, nuclear and cytoplasmic atypia of glandular epithelium, were identified only in the short-course group. DNA mutation analysis, using a panel of 56 genes frequently mutated in colorectal cancer, and p53 immunostaining were performed on both neoplastic and radiation-damaged non-neoplastic mucosa in a subset of short-course cases. Somatic mutations were identified only in neoplastic mucosa, supporting the concept that tissues with radiation-induced “dysplastic-like” features are not genetically transformed.

Conclusions: Pathologists should be aware of the characteristic morphological changes induced by radiation. The presence of features simulating dysplasia in the group treated with short-course radiotherapy may lead to serious diagnostic mistakes, if erroneously interpreted. NGS analysis further validated the morphological concept that radiation-induced abnormalities do not represent pre-neoplastic lesions.

(ITALIAN)

Background: La radioterapia preoperatoria con o senza chemioterapia si è dimostrata utile nel ridurre i tassi di recidiva locale e nel migliorare la sopravvivenza globale nel cancro del retto. I cambiamenti indotti dalle radiazioni nel tumoure sono ben descritti, mentre è stata prestata meno attenzione agli effetti delle stesse nella mucosa non neoplastica. Il nostro scopo è fornire un'analisi dettagliata delle caratteristiche morfologiche, immunoistochimiche e genetiche presenti nella mucosa non neoplastica. E' necessario che i patologi acquisiscano familiarità con le suddette caratteristiche morfologiche, quando valutano campioni di cancro del retto di pazienti trattati con radioterapia preoperatoria, in particolare con schema short-course, al fine di evitare diagnosi errate.

Metodi e risultati: Abbiamo confrontato 2 gruppi di 95 pazienti con cancro del retto trattati con radioterapia preoperatoria short-course (25 Gy somministrati in 5 giorni consecutivi, seguiti da intervento chirurgico pochi giorni dopo; 45 pazienti) o radioterapia long-course (45-50 Gy in 4-6 settimane, seguito da intervento chirurgico 4 settimane dopo; 50 pazienti). A seconda del tipo di protocollo, sono state osservate diverse caratteristiche istopatologiche, in termini di infiammazione, anomalie ghiandolari e differenziazione endocrina nella mucosa non neoplastica all'interno del volume irradiato. Da notare che le caratteristiche che imitano la displasia come la distorsione della cripta, l'atipia nucleare e citoplasmatica dell'epitelio ghiandolare, sono state identificate solo nel gruppo short-course. L'analisi della mutazione del DNA, utilizzando un pannello di 56 geni frequentemente mutati nel cancro del colon-retto, e l'immunocolorazione con p53 sono state eseguite sia su mucosa neoplastica che su mucosa non neoplastica e danneggiata da radiazioni in un sottogruppo di pazienti short-course. Mutazioni somatiche sono state identificate solo nella mucosa neoplastica, supportando il concetto che i tessuti con caratteristiche "displasiche" indotte dalle radiazioni non sono geneticamente trasformati.

Conclusioni: I patologi dovrebbero essere consapevoli dei caratteristici cambiamenti morfologici indotti dalle radiazioni. La presenza di caratteristiche che simulano la displasia nel gruppo trattato con radioterapia short-course può portare a gravi errori diagnostici, se interpretata erroneamente. L'analisi NGS ha ulteriormente convalidato il concetto morfologico secondo cui le anomalie indotte dalle radiazioni non rappresentano lesioni pre-neoplastiche.

2. Background

Colorectal cancers (CRCs) are gastrointestinal malignancies which develop either in the colon or the rectum [1]. According to their origin, they are defined as colon cancer or rectal cancer, although they are often joined, due to several biological and clinical common features [1]. Adenocarcinoma is the most frequent colorectal malignancy (up to 95% cases), followed by carcinoid tumours, gastrointestinal stromal tumours (GISTs), lymphomas and sarcomas [1,2].

Rectal cancer represents one of the most common malignancies in Western countries [3,4]. Radiation therapy is considered as standard treatment for locally advanced rectal cancer involving a high risk of local recurrence, when surgery represents the only treatment [5-10]. Two neoadjuvant regimens are preoperative long-course radiotherapy with concomitant chemotherapy (LCCRT) and preoperative short-course radiotherapy (SCRT), particularly recommended in tumours to be dealt by surgery [5-10].

LCCRT is advised in either low-seated tumours or bulky and unresectable ones, which should benefit from radiation-induced down staging [11,12]. LCCRT protocol includes 45–50 gray (Gy) in 4–6 weeks, followed by surgery four weeks later [11,12]. SCRT includes 25 Gy administration in five days in a row, shortly followed by surgery [11,12]. Preoperative radiation may lead to tumour regression by replacing neoplastic glands hit by fibrosis and inflammation [11,12]. Tumour regression is mainly found in LCCRT cases [11,12]. SCRT is not followed by significant tumour regression, as time frame from radiotherapy conclusion to surgery is too short to allow tumour down-staging [11,12].

Very few studies thoroughly analyzed histopathological features of radiation damage on normal colonic mucosa [13,14]. In accordance with the type of preoperative radiotherapy, different alterations may occur in normal colonic mucosa, as inflammation and glandular abnormalities are concerned [14]. We were struck by the finding that SCRT-associated morphological abnormalities may simulate dysplasia, thus causing possible diagnostic misinterpretation. Consequently, our study's design involved comparison between two groups of rectal cancer patients, either treated with SCRT or LCCRT. "Dysplastic-like" features in irradiated normal mucosa were observed only in SCRT specimens. On a subset of SCRT cases, we performed DNA mutation analysis of both tumour and mucosa with atypia, thus confirming that a "dysplastic-like" change was not a real dysplasia. Somatic mutations occurred only in tumours, suggesting that

tissues showing radiation-induced “dysplastic-like” features do not undergo genetic transformation.

Immunohistochemical p53 staining is deemed as substitute for mutational analysis [15-17]. Thus we performed p53 immunohistochemistry (IHC) in a SCRT subset, which had been evaluated through next generation sequencing (NGS) analysis. Further, we detected the presence of endocrine elements in benign and malignant tissues for both SCRT and LCCRT cases.

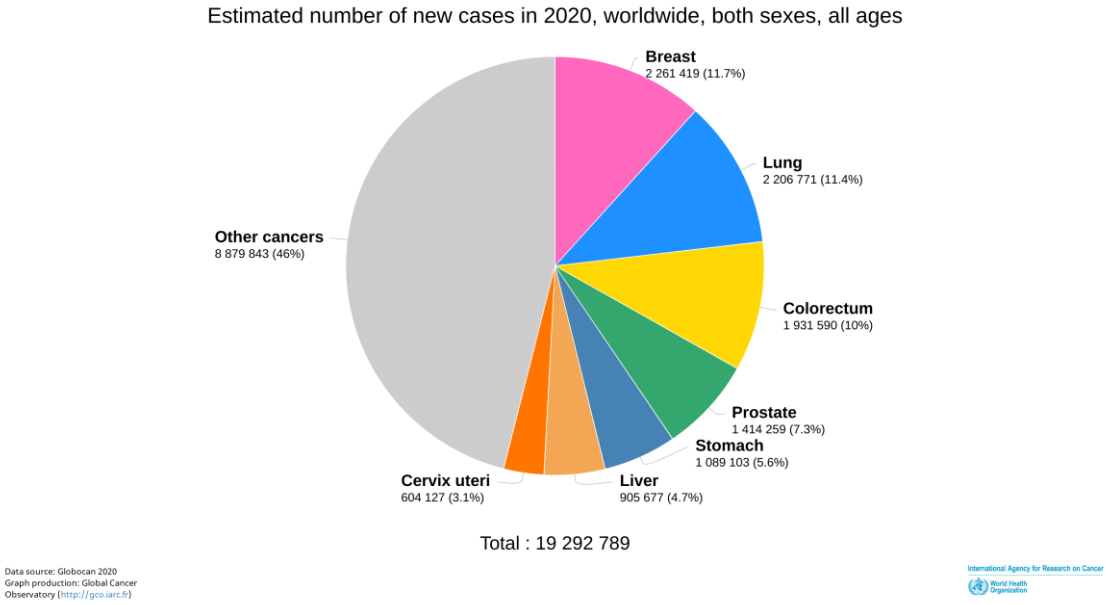
With the aim of allowing deeper understanding of our methods and results, I am going to introduce a first section of my thesis, which was entirely intended to typify epidemiological, histopathological, immunohistochemical, genetic features in addition to CRC management (particularly preoperative radiotherapy for rectal cancers).

3. Colorectal cancer epidemiology

3.1 General population CRC incidence

According to latest International Agency for Research on Cancer (IARC) statistics of World Health Association (WHO), CRC ranks third most frequent malignant disease (1.93 million new cases/year; 10% of total malignancies), only preceded by breast cancer (2.26 million new cases/year; 11.7% of total malignancies) and lung cancer (2.20 million new cases/year; 11.4% of total malignancies) (Figure 1) [18].

Figure 1. Estimated number of new cases in 2020, worldwide, both sexes, all ages [18]



CRC is third most common type of male malignancy (1.06 million new cases/year), after lung and prostate cancers (Figure 2), and second most frequent female malignancy (0.86 million new cases/year), after breast cancer (Figure 3) [19,20].

Figure 2. Estimated number of new cases in 2020, worldwide, males, all ages [19]

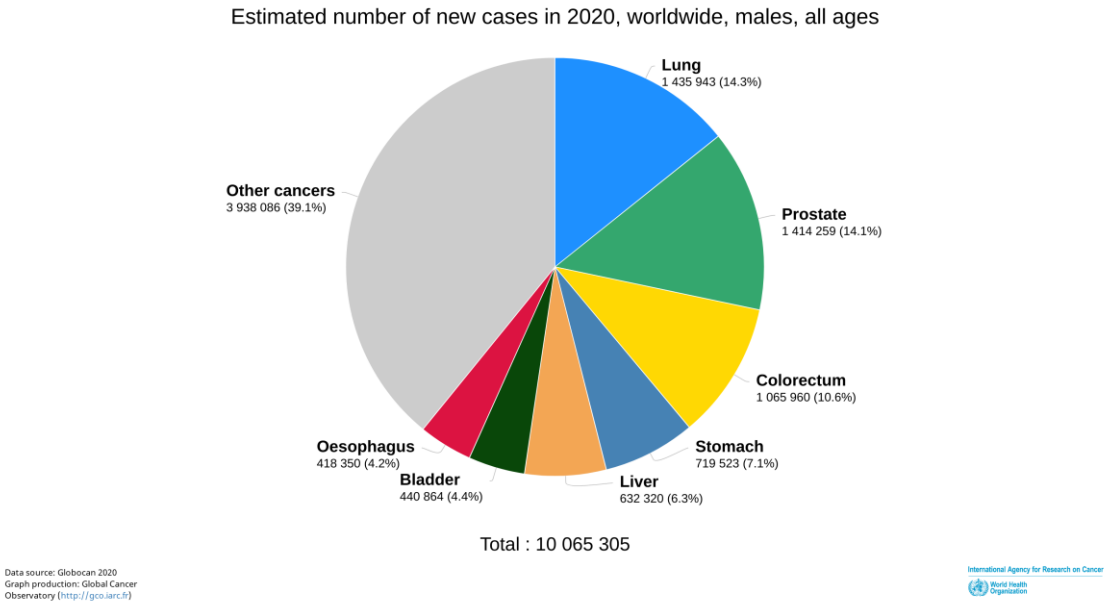
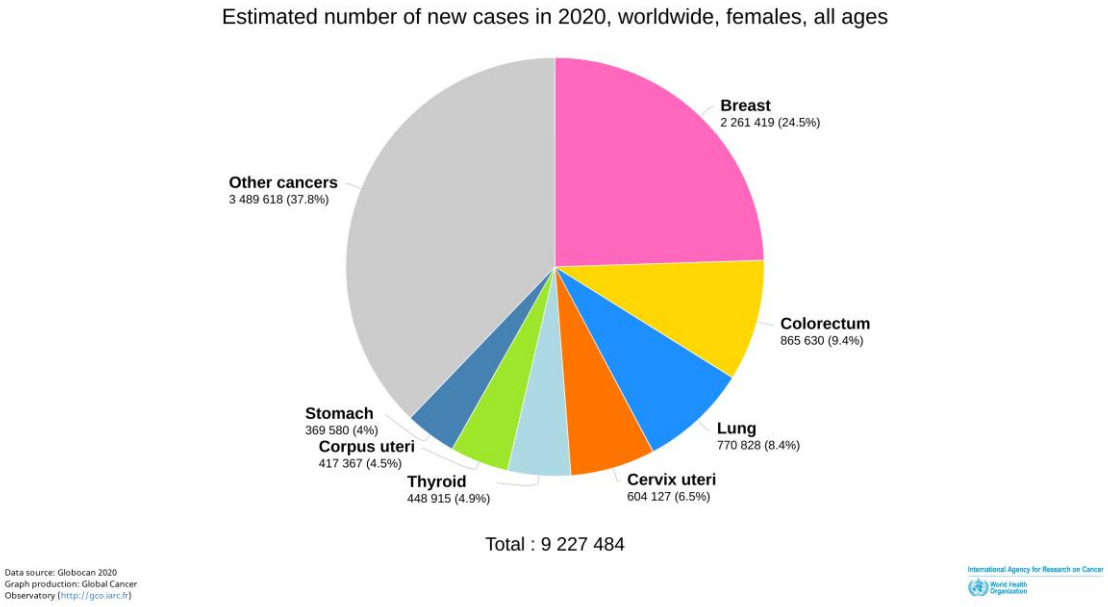


Figure 3. Estimated number of new cases in 2020, worldwide, females, all ages [20]



Provided that current forecasts are reliable, global CRC burden is expected to record 2.2 million new cases per year by 2030, thus recording a further 20% rise [21]. This increase is assumed to be result of environmental changes, higher level of sedentary lifestyle, increase in obesity, higher consumption of processed food, alcohol, meat consumption, and increased overall longevity [21].

In Australia and New Zealand, CRC incidence is higher (36.7 cases per 100,000) than Europe (28.8–32.1 cases per 100,000), Eastern Asia (26.5 cases per 100,000) and North

America (26.2 cases per 100,000) [21,22]. It records its lowest level in Africa (6.4–9.2 cases per 100,000) and South-Central Asia (4.9 cases per 100,000) [21,22].

All around the world, cumulative risk of developing CRC is 2.25% when aged between 0–74 years (2.71% in men and 1.83% in women), recording higher values in countries where human development index (HDI) is very high (3.42% between 0–74 years) rather than in countries with low HDI (0.84% between 0–74 years) [21,22].

Despite being more frequent in highly developed countries, such trend has recently become steady or it has even decreased, whereas an increased incidence has been recently detected in some low- and middle-income countries, that perhaps adopted western lifestyle [21,22].

The risk of CRC increases as people grow older, being more common in 50-year-olds or ever older [21,22]. Median age at diagnosis is 72 in women and 68 in men [21,22]. Notably, colon cancer cases are as follows: 1.5–5.4 per 100,000 people aged between 25–39 years (1.4–5.3 for men and 1.6–5.5 for women); 10.3–18.5 per 100,000 people aged between 40–49 years (10.2–18.9 for men and 10.4–18.1 for women); 34.3–62.5 per 100,000 people aged between 50–64 years (37.4–72.7 for men and 31.2–53.2 for women); 92.6–212.2 per 100,000 people aged between 65–85 years (107.7–229.7 for men and 79.2–200.4 for women); up to 240.9 in subjects aged 85 years or older (264.0 for men and 229.2 for women), thus recording an over 10-fold increase < 50 to >85 years [21,22].

As regards colon cancer, 1.097 million cases were diagnosed in 2018 (0.576 million men and 0.521 women), accounting for a 1.31% global cumulative risk between 0–74 years (1.51% in men and 1.12% in women) [21,22]. Cumulative risk of colon cancer is much higher in Australia and New Zealand (2.59% between 0–74 years), Europe (2.00–2.41% between 0–74 years) and North America (1.82% between 0–74 years), if compared to Africa (0.30–0.96% between 0–74 years) and South-Central Asia (0.29% between 0–74 years), in addition to countries with very high HDI (2.18% between 0–74 years) when compared to countries with low HDI (0.40% between 0–74 years) [21,22].

0.704 million rectal cancer cases were diagnosed in 2018 (0.430 million in men and 0.274 in women), accounting for a 0.91% global cumulative risk between 0–74 years (1.20% in men and 0.65% in women) [21,22]. Cumulative risk of rectal cancer is considerably higher in Europe (1.17–1.55% between 0–74 years), Australia in addition to New Zealand (1.40% between 0–74 years) and Eastern Asia (1.35% between 0–74 years) when compared to Africa (0.27–0.52% between 0–74 years) and South-Central

Asia (0.24% between 0–74 years), as well as in countries with very high HDI (1.30% between 0–74 years) when compared to those with low HDI (0.32% between 0–74 years) [21,22].

3.2 CRC incidence in young population

Since the early 1990s, both incidence and mortality have been decreasing among people older than 50, likely due to a combination of screening, shifts in distribution of risk factors (reduced smoking, wider use of aspirin) and improved treatments [23-25]. However, epidemiological studies demonstrated an alarming and continuing increased CRC incidence among people younger than 50 [24,25].

Early-onset CRC now accounts for 10-12% of all new CRC diagnoses [26]. Following adoption of population-based CRC screening in the 1990s, CRC incidence decreased by more than 35% in whole population [26]. Nevertheless, as opposed to marked decreases among older adults, CRC incidence among younger adults nearly doubled in the same time frame [26]. Incidence rates grew rapidly among 20–49 year olds in the USA, shifting from 8.6 per 100,000 in 1992 to 13.1 per 100,000 in 2016, with the most significant increases taking place among 40–49 years olds [26].

Such increases in early-onset CRC were reported in Western countries, including Canada, Australia, UK, and Asia. Despite overall population trends in aging, by 2030 approximately 11% colon cancers and 23% rectal cancers are going to affect adults younger than 50 [25].

Early-onset CRC has increased through subsequent birth cohorts, while people born in 1960 or later are at higher risk of CRC, if compared to older generations [24,27]. In the USA, CRC incidence rates are higher among 40-year-olds, who were born in 1970 (24.4 per 100,000) than among 40-year-olds who were born in 1950 (18.3 per 100,000) [26].

To our surprise, increased early-onset CRCs by birth cohort occurred worldwide, although CRC risk differed by birth cohort in each country [24,27]. In the USA, incidence rates started to increase among *Baby Boomers*, while they are presently at highest level among *Generation X* [24,27]. In Canada and Australia, increased incidence of colon cancer vs rectal cancer was first recorded among people born in the 1970s [28,29]. In Asian populations from Japan, Hong Kong and Shanghai, increased incidence rates were recorded in later birth cohorts [30]. Birth cohort effects point out exposures either occurring in early life or frequently experienced by younger generations, who may undergo an increased risk of early-onset CRC [31].

If compared to older patients who are mostly affected by proximal colon tumours, a higher percentage of younger patients suffer from distal colon or rectum primary tumours [26].

Increasing early-onset CRC incidence rates were driven by increases in rectal cancer if compared to colon cancer, particularly in white population [32]. From the early 1990s to 2016, cases of rectal cancer increased by more than 90% (2.6 to 5.1 per 100,000) across all racial/ethnic groups, compared to a 40% increase in colon cancer cases [26]. Women witnessed the most important relative increase in rectal cancer incidence (2.7 to 4.6 per 100,000) [26].

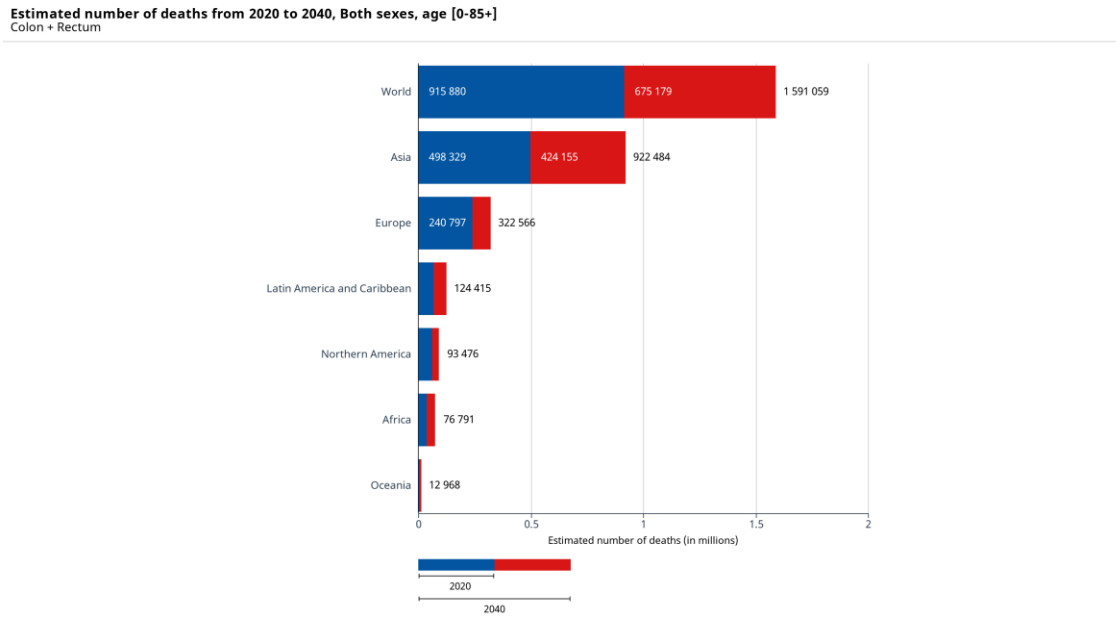
A recent meta-analysis by Din *et al* examined 40 studies stemming from 12 countries across five continents [33]. The research focused on temporal trends in yCRC incidence and prevalence [33]. Altogether, the research found an increasing yCRC incidence with a + 1.33 worldwide pooled Annual Percent Change incidence (APCi) (95% CI, 0.97 to 1.68; $p < 0.0001$) [33]. That was largely driven by increasing incidence in the USA, Australia, and Canada with reported overall APCi's up to + 7.9 (95% CI, 1.1 to 15.1) and nearly a 30% increased incidence over 20 years [33]. Relying on comparatively fewer studies and inconsistent findings, similar conclusions cannot be drawn from European, Asian and African studies [33]. According to Din *et al* systematic review, trends of yCRC increasing risk seem to be driven by rectal cancers, as outlined by 9 out of 14 studies, that specifically evaluated it and with APCi's up to + 4.03 ($p < 0.001$) [33]. In a previous meta-analysis, O'Connell *et al* highlighted rectum and sigmoid colon as the most commonly affected sites (54% tumours) [34]. This result agrees with Din *et al* showing that, at population level, rectal cancer contributes to increased yCRC incidence [34]. Noteworthy, O'Connell *et al* found no difference in yCRC sex distribution (48.6% in women and 51.4% in men) while according to Din *et al*, pooled sex-specific APCi's were similar for women (+ 1.02; 95% CI, 0.20 to 1.83) and men (+ 0.99; 95% CI, 0.31 to 1.67) [34].

3.3 Mortality

In accordance with IARC mortality database last update, worldwide CRC deaths have approximately reached 0.915 million in 2020, accounting for 9.19% of cancer-related ones [35]. In particular, colon cancer represents the 5th deadliest cancer with 576,858 deaths estimated in 2020, including 5.79% of all cancer deaths. Meanwhile, rectal cancer is the 10th deadliest one, with 339,022 deaths, accounting for 3.4% of all cancer

deaths. Provided that current trend is not reversed, by 2040 CRC mortality will record 1.591 million deaths per year, with a further 73.7% increase [35].

Figure 4. Estimated number of deaths from 2020 to 2040, Both sexes, age [0-85+] [35]



Taking into account particular geographic areas, 2040 CRC mortality will record 0.922 million deaths per year in Asia, with a further 85.1% increase, 0.322 million deaths per year in Europe (+34%), 0.124 million deaths per year in Latin America and Caribbean (+82.8%), 0.093 million deaths per year in Northern America (+49.6%), 0.076 million deaths per year in Africa (+97.8%), and 0.012 million deaths per year in Oceania (+74.9%) [35].

Taking into account HDI areas, 2040 CRC mortality will record 0.778 million deaths per year in high HDI, with a further 87.2% increase, 0.124 million deaths per year in medium HDI (+77.2%), and 0.049 million deaths per year in low HDI (+103.5%) [36].

3.4 Trends and Survival outcomes

The relationship between trends in CRC incidence and mortality can be expressed thanks to 3 well separated global groups [22,37]. The first one, which includes medium-HDI countries such as Brazil, Russia, China, Latin America, the Philippines and the Baltic countries, witnessed both increased incidence and mortality rates, in the past decade [22,37]. Above mentioned countries are now undergoing an economic transition, which is the possible cause of an increased CRC incidence. The second group, mostly including high-HDI countries such as Canada, the United Kingdom (UK), Denmark and

Singapore, witnessed both an increased incidence and a decreased mortality, due to improved treatment options [22,37]. Lastly, the third group which includes countries with highest HDI (such as the USA, Iceland, Japan, and France) recorded a drop in both mortality and incidence, due to successful preventive measures and treatments [22,37]. In the second and third above mentioned groups, amended CRC treatments led to decreased CRC mortality, even against increased incidence [22].

Polyps removal and other early detection methods, colonoscopies, flexible sigmoidoscopies, computed tomography (CT) colonography, faecal immunochemistry and faecal occult blood testing have represented drive for longer survival times [22,38]. At first, the introduction of better screening tests may have increased incidence rates, thanks to the possibility of diagnosing previously undiagnosed diseases [22,37]. In the long term, it also reduced mortality by removing pre-cancerous or un-metastasised polyps [22,37].

USA belong to third group of highest HDI countries, as they recorded decreased CRC incidence and mortality [22]. In the USA, 5-year relative survival rate for stage I colon cancer was approximately 92%, while rates for stage IIA and stage IIB reached 87% and 65% [22]. To our surprise, the 5-year survival rate for stage IIIA and stage IIIB recorded slightly higher values, 90% and 72% respectively [22]. Stage IIIC had a 53% survival rate, while stage IV or metastatic colon cancer recorded a 12% 5-year survival rate [22]. Five-year survival rates for rectal cancer were mostly slightly lower, with 88% for stage I; 81% for stage IIA; 50% for stage IIB; 83% for stage IIIA; 72% for stage IIIB; 58% for stage IIIC; 13% for stage IV [22].

4. Colorectal cancer histopathological features

More than 90% CRCs belong to adenocarcinomas originating from epithelial cells in the colorectal mucosa [2]. Other uncommon CRCs include the following ones: neuroendocrine, mucinous, signet ring cell, medullary, micropapillary, serrated, cribriform comedo-type, adenosquamous, spindle cell, and undifferentiated [2]. Conventional adenocarcinomas are marked by glandular formation, which represents the basis for histologic tumour grading [2]. In well differentiated adenocarcinomas, >95% tumour is gland forming (Figure 4) [2]. Moderately differentiated adenocarcinomas show 50-95% gland formation (Figure 5) [2]. Poorly differentiated adenocarcinomas are mainly solid ones with <50% gland formation (Figure 6) [2]. Most colorectal adenocarcinomas (~70%) are diagnosed as moderately differentiated ones [2]. Well and poorly differentiated carcinomas account for 10% and 20%, respectively [2].

Figure 4. Well-formed neoplastic glands consistent with well differentiated adenocarcinoma (G1) [Dr. Magda Zanelli; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]

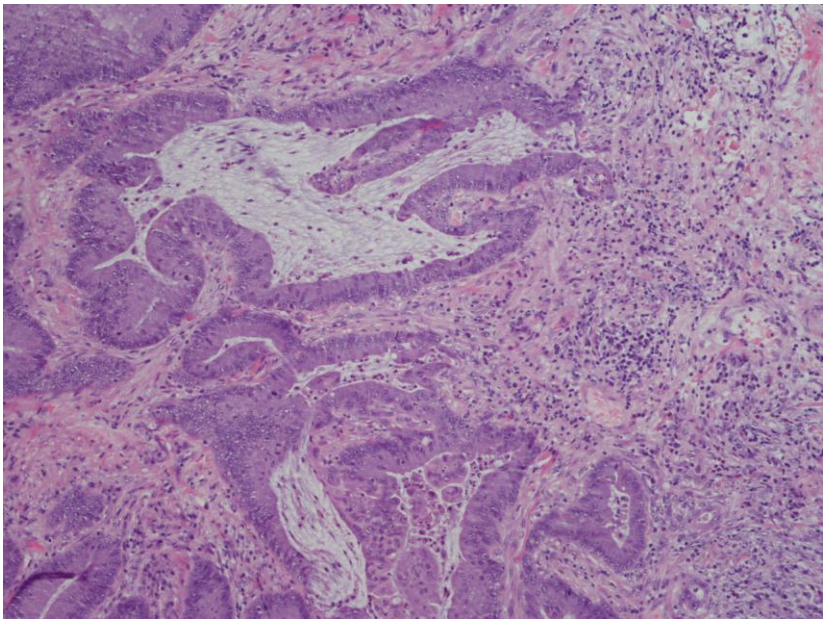
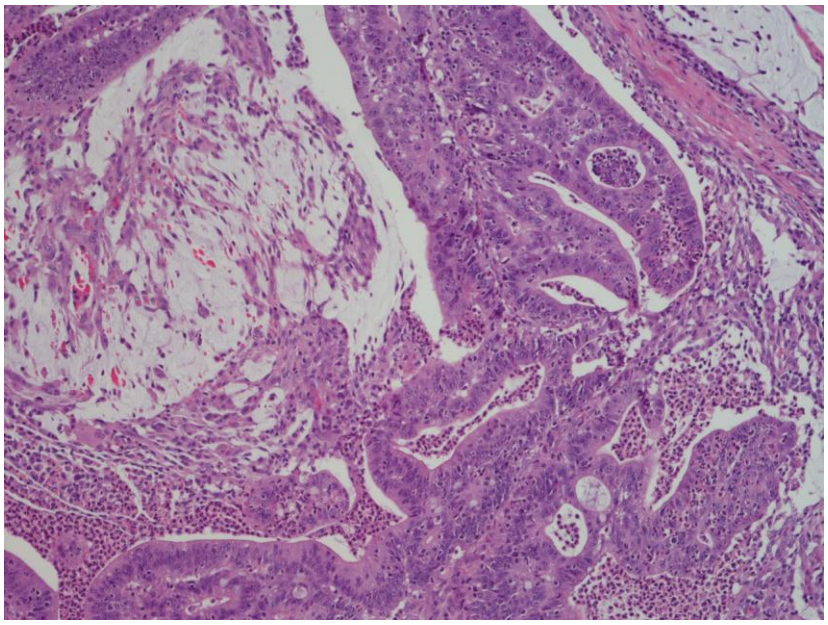
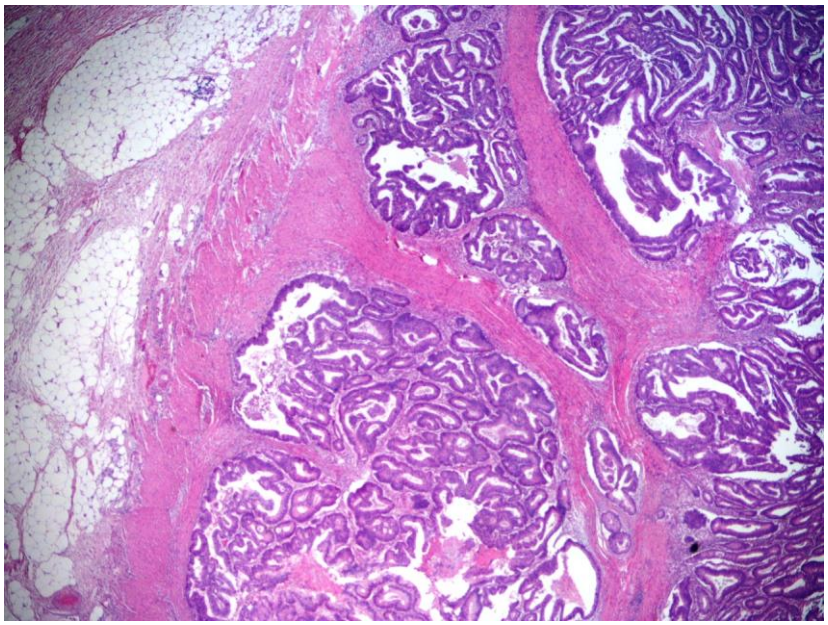


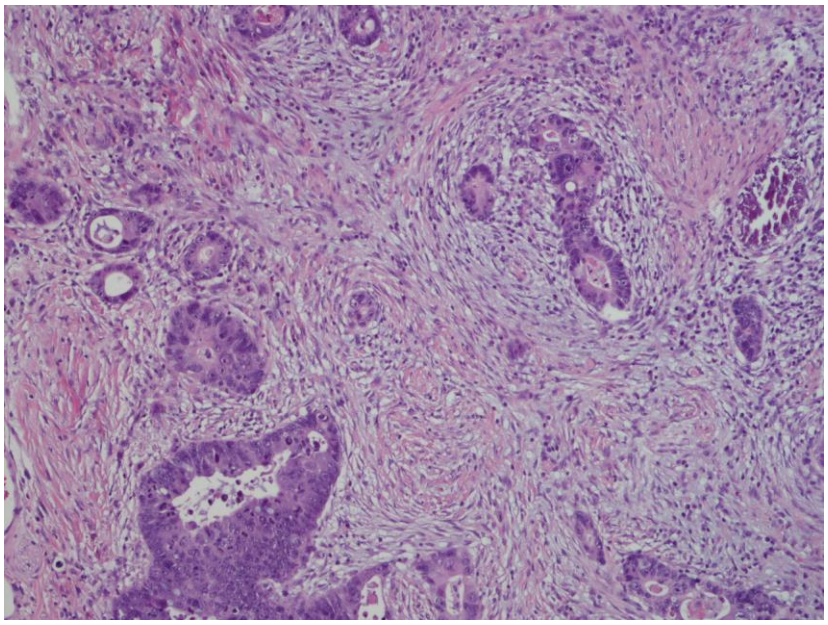
Figure 5. A Cribriform neoplastic glands of moderately differentiated adenocarcinoma. Lakes of mucus are visible on the left; **B** Moderately differentiated adenocarcinoma with cribriform neoplastic glands; **C** Neoplastic glands of different sizes within a desmoplastic stroma (moderately differentiated adenocarcinoma). Some glands show pseudostratified epithelium; **D** Moderately differentiated adenocarcinoma with mucinous differentiation; **E** Neoplastic glands with cribriform features (moderately differentiated adenocarcinoma) [Dr. Magda Zanelli; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



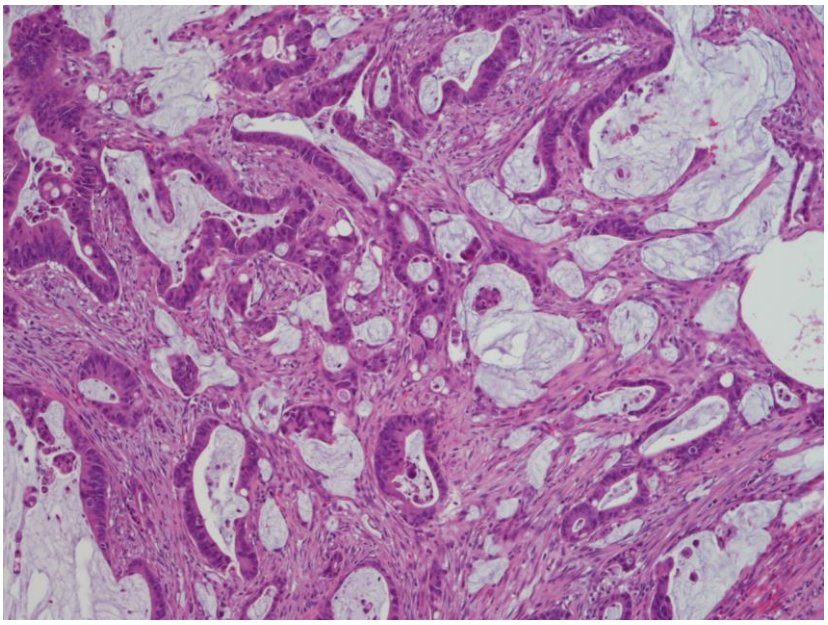
A



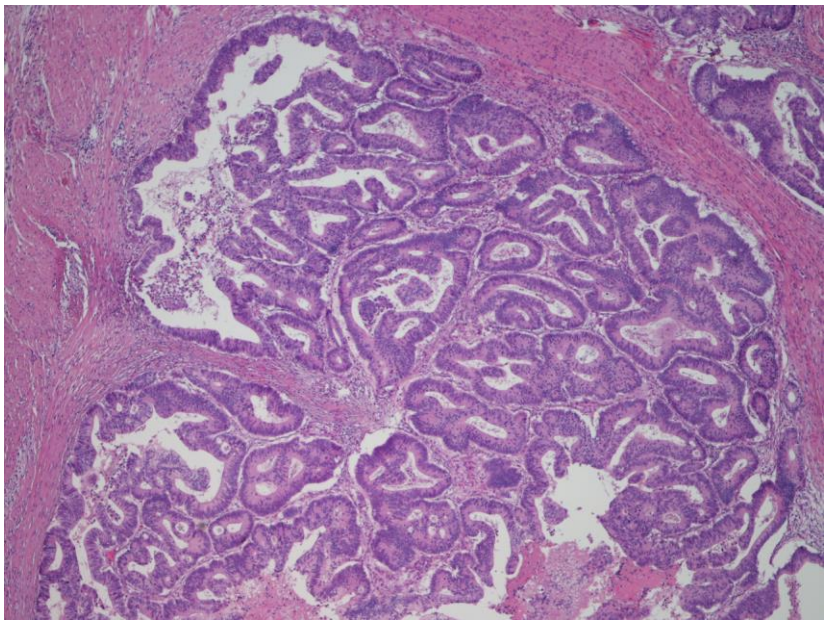
B



C

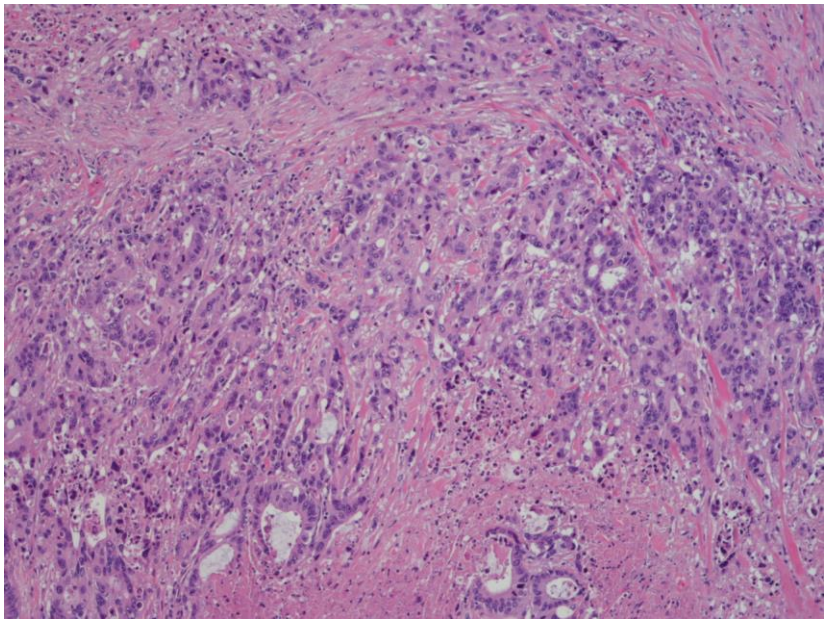


D

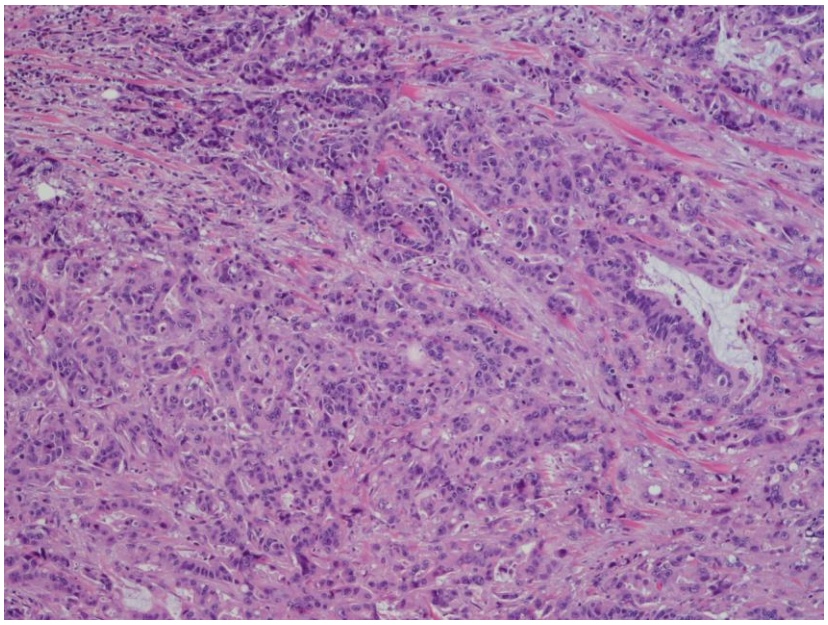


E

Figure 6. A Fused neoplastic glands and single cell pattern of growth are indicative of poorly differentiated adenocarcinoma (G3); **B** The glandular structures are difficult to be recognized, as glands are almost completely fused (poorly differentiated adenocarcinoma) [Dr. Magda Zanelli; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



A



B

4.1 Macroscopic features

Approximately 50% of all carcinomas develop in the rectosigmoid area, although their relative incidence seems to record a decrease [39]. Past decades have been recording a space shift toward the proximal colon [40]. At presentation, right-sided tumours are associated to older age [41]. Multicentric carcinomas are detected in 3%-6% cases [42].

Broadly speaking, most colorectal carcinomas are either polypoid or ulcerative/infiltrating ones [43]. The former looks like a bulky mass with well-defined rolled margins, and a sharp dividing line with the normal bowel [43]. The latter has a less elevated surface and is centrally ulcerated [43]. A particular variant of this tumour type, which has been thoroughly described by Japanese literature, is referred to as “flat” or “depressed” carcinoma and is thought to arise de novo, rather than through adenoma malignant transformation [43]. These flat carcinomas have a greater tendency for deep invasion with lymphovascular permeation than the more common polypoid types [44].

All in all, there is good match between tumour gross and microscopic margins [45]. Colorectal tumours rarely show extensive lateral superficial spreads as we can often detect in stomach, although it sometimes occurs [45]. By examining cross section, grayish white tissue replaces the bowel wall [45]. The edges may be well marked or create finger-like projections extending from the main mass [45]. Highly mucinous tumours show a gelatinous, glaring appearance, while layers of mucus may separate the layers of bowel wall [45].

At gross examination, it is paramount to assess: whether tumour is confined to wall or has extended to pericolic tissues; whether gross invasion of veins is taking place, and remaining colon shows any other carcinomas or polyps.

4.2 Microscopic features

Well differentiated adenocarcinomas and moderately differentiated ones, which secrete variable amounts of mucin, represent the common malignant tumour type affecting large bowel [46].

The tumour cells represent a combination of columnar and goblet cells, with random presence of neuroendocrine cells and exceptional occurrence of Paneth cells [46]. The carcinoma consistently elicits an inflammatory and desmoplastic reaction, which is particularly prominent at tumour edge [46]. Most inflammatory cells are T lymphocytes, although B lymphocytes, plasma cells, histiocytes, and S-100 protein–positive dendritic cells are also present [47,48]. By chance, many eosinophils might be present, which could be due to interleukin-5 production [49].

Tumour surface may have a papillary or villous configuration, which must be distinguished both from previously described residual villous adenoma and micropapillary pattern [50]. Tumour may invade all bowel layers, extend into pericolic fat, permeate perineurial spaces, thus invading veins [50]. The latter feature, which is of prognostic significance, can be better appreciated with stains for elastic fibers (Verhoeff–van Gieson or Movat stain) [50].

Evidence of invasion is the key of microscopic examination [2]. When muscularis mucosae can be identified, determining whether it is disrupted by neoplastic cells is of paramount importance [2]. Invasive carcinoma peculiarly invades through the muscularis mucosae into the submucosa, and it is sometimes seen close to submucosal blood vessels [2]. Presence of desmoplasia or desmoplastic reaction, which is a fibrous proliferation surrounding tumour cells secondary to invasive tumour growth, represents an important feature of invasion [2]. Invasive colorectal carcinoma also frequently shows characteristic necrotic debris in glandular lumina, so-called “dirty necrosis”, which can be quite useful in suggesting colorectal primary, in case of metastasis of unknown origin [2].

Tumour edge may show foci of a residual adenoma [51]. More commonly, a reactive hyperplastic alteration may be detected in the glands, being taller, more tortuous, and

marked by more frequent goblet cells than normal mucosa (so-called transitional mucosa) [51].

4.3 High grade dysplasia

Pathologic assessment of an adenomatous polyp and dysplasia means to determine whether high grade dysplasia is present or not, as it represents outright forerunner to invasive colorectal adenocarcinoma [2]. High grade dysplasia shows as a constellation of architectural complexity and cytological atypia, that are more malignant-appearing than those seen in a conventional adenoma [2]. From architectural point of view, high grade areas show typical increased glandular density with crowded glands, that have a cribriform or back-to-back growth pattern [2]. From a cytological point of view, cells with high grade dysplasia exhibit rounded nuclei, coarse chromatin, prominent nucleoli and loss of nuclear polarity, while nuclei have no longer perpendicular orientation to the basement membrane [2]. Necrotic debris within the lumina of dysplastic glands may be detected [2].

High grade dysplasia is usually focal and located on the surface polyp portion, thus requiring no additional treatment rather than polypectomy, when polyp undergoes a complete endoscopic resection [2]. High grade dysplasia in the colorectum means carcinoma in situ or intraepithelial carcinoma [2].

Intramucosal adenocarcinoma, which is defined by lamina propria invasion including invasion into muscularis mucosae (but not through it), still belongs to high grade dysplasia due to its negligible potential of metastasis and it can still be successfully treated just by polypectomy [52].

5. Colorectal cancer histochemical and immunohistochemical features

In a histochemical perspective, most colorectal carcinomas are positive for mucin stains [53]. Immunohistochemically, MUC1 and MUC3 are the main mucin protein cores expressed by conventional colorectal adenocarcinoma (as opposed to MUC2 in mucinous carcinoma) [53]. There is also expression of MUC13, particularly in poorly differentiated tumours [54]. Recently, the serrated pathway of CRC has witnessed an association with increased expression of MUC2, MUC5AC, and MUC6 [55].

Colorectal adenocarcinomas are invariably positive for cytokeratin (CK), while CK20 positivity and CK7 negativity represent the most common features [56]. Reverse pattern, which is useful in telling colorectal adenocarcinoma from other ones (such as lung and ovary ones) is hardly found [57]. However, aberrant patterns of immunoreactivity (such as CK7 positivity) can be detected in some colorectal carcinomas, in particular the poorly differentiated ones [58,59]. Carcinoembriogenic antigen (CEA) reactivity is also the rule while failure to demonstrate CEA in adenocarcinomas makes a colorectal origin unlikely [60]. The positivity for this marker is equally distributed throughout the cell surface in most cases, as opposed to polar distribution observed in normal mucosa and in better differentiated tumours [61]. Many monoclonal antibodies to different epitopes of the CEA molecule are available, although antibodies that recognize group1 or group2 epitopes offered the highest levels of sensitivity and specificity [56].

Caudal-type homeobox CDX2 gene encodes a transcription factor that plays an important role in intestinal epithelial cell proliferation and differentiation [62]. It is found by immunohistochemistry in most colorectal carcinomas, but it can also be expressed in primary mucin-producing carcinomas of ovary, bladder, and lung, as well as pancreaticobiliary adenocarcinomas [62,63].

Tumour-associated glycoprotein 72 (TAG-72), which is recognized by monoclonal antibody B72.3, shows in virtually all invasive colorectal carcinomas but also most hyperplastic and adenomatous polyps and even in normal mucosa [64]. Nevertheless, reactivity frequency and expression pattern vary according to condition [64].

Regardless of differentiation, markers which are consistently expressed by colorectal carcinoma are: villin (a cytoskeletal protein associated with the axial microfilament bundles of brush border microvilli); cathepsin B (a lysosomal cysteine proteinase); neuropilin-1 (a molecule normally present in the developing nervous system); SRCA2

(an ATPase crucial to many cell functions); cadherin-17 (also known as liver-intestine cadherin) [65-68]. Calretinin can be expressed by a minority of colorectal adenocarcinomas (especially the undifferentiated ones), that must be kept in mind in the differential diagnosis with mesothelioma [69].

Most colorectal carcinomas show immunoreactivity for human chorionic gonadotropin (hCG) [70]. This feature seems particularly common in mucinous and poorly differentiated tumours [71]. Estrogen and progesterone receptors are either absent or present in a small minority of tumour cells [72]. Racemase, a marker for prostatic adenocarcinoma, is expressed in over half of colorectal adenocarcinomas, representing a potential source of misdiagnosis [73].

6. Colorectal cancer genetic features

CRC represents a genetically and cellular heterogeneous disease [74]. The tumour mass is made up of both altered epithelial cells, that accumulate driver mutations, and tumour microenvironment made up of stromal and immune cells [74]. In a morphological perspective, two morphological multi-step pathways are represented by traditional adenoma-to-carcinoma sequence and serrated adenoma pathway [74]. Genetic studies have detected two major molecular pathways: *chromosomal instability (CIN)* pathway and *microsatellite instability (MSI)* one [74]. Some authors regard *CpG island methylator phenotype (CIMP)* as a third molecular pathway although it overlaps with MSI positivity, according to TCGA [75,76].

Tumour malignancy must experience three steps, i.e. breakthrough, expansion, and invasion, taking 2 to 3 decades and requiring at least 2 to 3 driver gene mutations [77]. A common mature cell cannot suddenly turn into a cancer cell [77]. CRC arise in the stem cell compartment in colonic crypts and CRC lifetime risk is directly related to total lifetime number of stem cell divisions [78].

First driver gene mutation provides the cell with a small survival advantage, resulting in a proliferating hyperplastic lesion with an increased risk of a second driver mutation [77,78]. The second driver gene mutation provides the cell with autonomy, uncontrolled self-renewal and immortal characteristics paving the way for a third driver mutation [77,78]. Third driver gene mutation allows the cell to invade surrounding tissues [77,78]. This mutation also provides tumour with capability to metastasize, while no additional driver-mutations are required for dissemination [77,78].

A driver gene mutation causes a small increase in cell birth/cell death ratio and an approximately 0.4% growth advantage in cellular life span, including proliferation capability under limited availability of nutrients [77]. By repeating replications once or twice a week over 2 to 3 decades, such growth advantage translates into a slow but exponential increase of tumour cell number [77].

Passenger mutations point to mutations that do not allow any growth advantage, but happen to be inside the cell when it acquires driver gene mutation [77]. Most passenger mutations are detected in noncoding DNA without showing any clinical relevance [77]. 99.9% mutations and epigenetic alterations found in malignant tumours have no impact on neoplasia itself [78,79].

Driver gene mutations represent rare events, even in tumours, whose growth is usually curbed by host defenses, lack of nutrition, and hypoxia, due to abnormal vasculature [77].

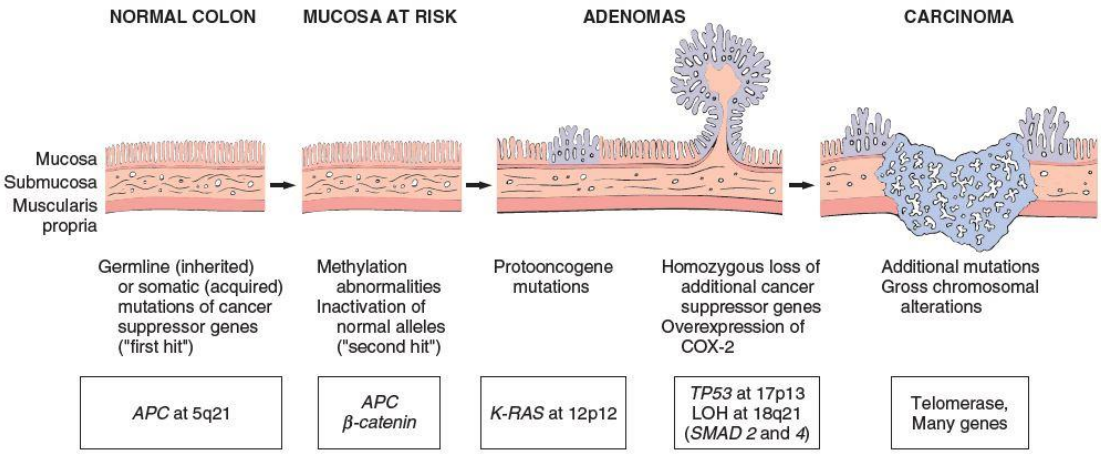
The order of mutations is essential for malignant transformation [77]. In CRC, APC mutation must take place before KRAS mutation for carcinogenesis to progress [77]. When mutations arise differently, adenoma is not going to turn into carcinoma (as it happens in most adenomas) [77]. In addition, consequences of driver mutation are related to frame work, where it arises [77]. Targeting identical BRAF mutation both in melanoma and CRC shows striking regression in the former tumour but no effect in the latter one [77]. Such consequence is due to epithelial growth factor receptor (EGFR) expression in CRC circumventing BRAF inhibitors [77].

6.1 Chromosomal instability (CIN) pathway

In a recent study looking at CRC mutations, 1321 genes associated with human cancers were analyzed in 468 colorectal tumours [80]. A subset of 17 genes with similar mutation frequencies most commonly mutated in CRC were identified: KRAS, TP53, APC, SMAD4, FBXW7, BRAF, TCF7L2, PIK3CA, GNAS, CBX4, ADAMTS18, TAF1L, FAM123B, CSMD3, ITGB4, LRP1B, and SYNE1, given in decreasing order of mutation rate [80].

Six of these genes have been classified as CRC driver genes: APC, KRAS, BRAF, PIK3CA, SMAD4, TP53 with APC, KRAS, PIK3CA, and p53 being most frequently mutated (Figure 7) [77,80]. APC, KRAS, and BRAF mutations are early events in the transition from normal epithelium to adenoma, whereas PIK3CA mutation and loss of SMAD4 and p53 (due to mutations or epigenetic silencing) are late events enabling tumour cells to invade surrounding tissues and metastasize thereby transforming the adenoma into a carcinoma [77,80].

Figure 7. Chromosomal instability (CIN) pathway



The APC mutations usually co-occur with either KRAS or TP53 mutations, or both [77,80]. This triumvirate is highly lethal and predicts poor outcome, whereas BRAF, ITGB4, CBX4, CSMD3, SYNE1, FBXW7, and TAF1L are strongly correlated with MSI but not with metastatic disease [77,80].

APC (adenomatous polyposis coli) gene. It is considered the gate keeper gene for CRC and mutations are found in 80% of all cases [77,81]. Mutations may occur in different zones of the gene and more than 1 mutation can occur simultaneously [77]. These mutations are frameshift mutations causing truncation of the APC protein, preventing it from binding β-catenin to the cytoplasmic domain of the membranous e-cadherin complex, which becomes defect [77]. Instead, free cytoplasmic β-catenin molecules translocate to the nucleus increasing signaling through the Wnt pathway [77].

APC plays a central role in predicting overall survival [77,80]. As mentioned, APC may assume one or more truncating mutations, each with a striking impact on survival [77,80]. Tumours with more than 1 APC mutation (>30% of CRC) concurrent with mutant KRAS and loss of TP53 confer the poorest survival among subgroups examined [77,80].

KRAS (Kirsten rat sarcoma viral oncogene homolog). Mutations in this oncogene are early events in CRC tumourigenesis occurring in approximately 30% to 40% patients [82]. Being a downstream component of EGFR signaling network, gene product K-ras controls growth-promoting signals from cell surface to nucleus [77,82]. To transmit signals, K-ras must be activated by binding to GTP. Active GTP-bound K-ras interacts with more than 20 effector proteins, while switching to active form normally takes place when growth factor receptors (such as EGFR) are activated [77,82] EGFR governs cancer cell proliferation, apoptosis and tumour-induced neoangiogenesis [77,82]. When

KRAS mutations take place, K-ras protein remains functionally active, regardless of EGFR, leading to constitutive activation of downstream pathways, including Ras/Raf/MAP/MEK/ERK and PI3K pathways [83,84].

KRAS mutation and subsequent EGFR-independent activation of downstream pathways predict resistance to treatment with anti-EGFR monoclonal antibodies, such as cetuximab or panitumumab, being limited to a subset of patients with metastatic disease and wild type KRAS [77].

KRAS mutation impact on outcome has been controversial [85]. Two recent analyses on metastatic CRC have reported lack of association with KRAS mutations, time-to-recurrence, recurrence pattern or overall survival [85].

BRAF (B-raf proto-oncogene). BRAF, which encodes a protein called B-raf, belongs to raf/mil family of serine/threonine protein kinases [77,86]. B-raf is activated by RAS GTPase (eg, K-ras) and plays an important role in the EGFR-mediated MAPK pathway affecting cell growth, proliferation, differentiation and other key cellular processes, such as cell migration, apoptosis (through BCL-2 regulation) and survival [86].

Among European and Northern American populations, BRAF mutations are the least frequent mutations as they occur in less than 15% CRC cases [77,87]. BRAF-mutated tumours are often right-sided, more frequent in woman, and of higher grade [87]. BRAF mutations are associated with poor prognosis, sporadic MSI, female subjects and old age, while they aren't ever found in hereditary nonpolyposis CRC [87].

Unfortunately, CRC patients have a poor answer to BRAF inhibitors, due to EGFR expression circumventing the effect [88].

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha). It encodes PI3Ka, a kinase playing a key role in regulating cell proliferation, survival and motility [77]. Occurring in approximately 30% CRCs, PIK3CA mutation leads PI3Ka to signal without regulation, leading to a proliferation spinning out of control [77].

Amino acid glutamine is often required for cancer cell growth [77,89]. Until recently, PIK3CA mutations have not been known as "replanners" of cancer cell metabolism [77,89]. In CRC cells, PIK3CA mutation replans glutamine metabolism by upregulating glutamate pyruvate transaminase 2 (GPT2), making tumour cells more dependent on glutamine [89]. Glutamate pyruvate and glutamine play key roles in intermediary metabolism, replenishing citric acid cycle [89].

SMAD4 (SMAD family member 4). Encoded SMAD4 protein is a tumour suppressor and critical regulator of TGF-beta pathway controlling proliferation [77]. Cells subject to

SMAD4 mutations may proliferate out of control [77]. Moreover, mutation is strongly involved in EMT and metastatic process [77]. Although it stresses TGF-b signaling, SMAD4 activity is regulated by Wnt and fibroblastic growth factor and turns out as not compulsory for constitutive activation of TGF-b pathway [90].

SMAD4 mutation is detected in 10%-20% CRC patients and it also predicts resistance to oxaliplatin-based chemotherapy, thus leading to poor prognosis [91,92].

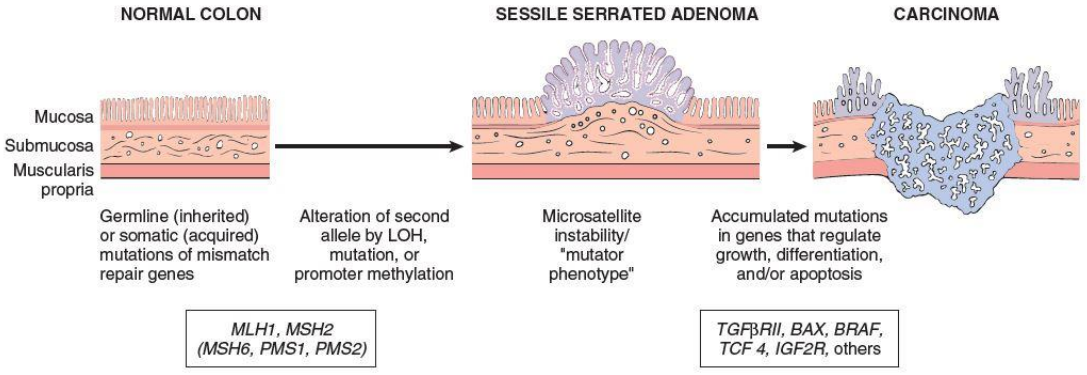
TP53. Impairment of cell cycle progression (in case of DNA damage) and promotion of repair represent the two most significant effects of this tumour suppressor gene [77]. When DNA damage is too extensive and repair cannot take place, cell undergoes apoptosis [77]. As mutant p53 protein has lost its growth-inhibitory function and is quite stable, it may even dominate over any remaining wild-type p53 having extremely short half-life [77].

Mutations or epigenetic silencing leading to loss of p53 function frequently happen in most cancers and are detected in nearly 60% CRC [93]. Such loss of function means increased cancer aggressiveness, in addition to higher recurrence and mortality rates [94].

6.2 Microsatellite instability (MSI) pathway

Although not being classified as driver gene mutation, detection of mutations and epigenetic changes in mismatch repair (MMR) genes is a key in CRC natural course (Figure 8) [77]. In humans, 9 MMR function genes have been identified, out of which 5 gene mutations have a special role, as they lead to Lynch syndrome [77]. Genes and frequency where they are mutated are as follows: MLH1 (49%), MSH2 (38%), MSH6 (9%), PSM2 (2%), and PMS1 (0.3%) [77].

Figure 8. Microsatellite instability (MSI) pathway



Cancers arising in cells with defective MMR gene function show a peculiar accumulation of replication errors in DNA microsatellites [95]. Microsatellites are small repeating stretches of DNA scattered throughout entire genome (1–6 base pairs) [77]. They account for approximately 3% of human genome and record high mutation rates [77]. When compared to normal tissue, any inconsistency in the number of microsatellite nucleotide repetitions is defined as microsatellite instability (MSI), which comes from a failure of MMR system to correct DNA base errors and keep genomic stability [77,95].

In sporadic CRC without MMR mutations, 3 to 6 driver mutations may take place, in addition to multiple passenger mutation, while CRC with MMR deficiency records a level of mutations which is 10 times higher [77,95]. Apart from mutations, silencing of MMR genes may be due to hypermethylation, resulting in low or lacking production of MMR gene products, which may be detected in sporadic CRC (usually by silencing of MLH1) [77,95].

MSI examination is significantly related to treatment with immune checkpoint inhibitors, as they are very effective in subsets of CRC patients with significant MSI (MSI-H) [77,95,96].

MSI is high (MSI-H) when 2 or more markers out of 5 ones in core panel show instability or when more than 30% markers record instability in other marker panel [96]. MSI is low (MSI-L) when 1 marker out of the 5 ones in core panel shows instability or fewer than 30% markers show instability in other marker panels. MS is stable (MSS) if 0 (or 0%) of markers show instability in the core panel or other marker panels [96].

6.3 Complementary pathways to sporadic CRC

CDX2 has proved to be main gene regulator of gene expression in intestinal epithelial cells [97]. No surprise that aberrant expression of CDX2 affects homeostasis of colonic epithelial cells [97]. In particular, absence of CDX2 expression and presence of BRAF mutations are associated with serrated pathway to colon cancer [98]. In the cohorts under examination, loss of CDX2 expression was predominantly found in less than 10% cases [99].

Loss of CDX2 expression is rarely due to mutations in CDX2 gene, although it represents a consequence of promoter methylation in cancer cells [100]. Reduced CDX2 expression was reported to be related to cancer recurrence, while stage II CRC patients

with lack of CDX2 expression took advantage from adjuvant chemotherapy [101]. In some CRCs, CDX2 overexpression has been reported as a consequence of gene duplication event of CDX2 gene [102]. In such cases, CDX2 plays a key role as linage survivor gene for cancer cells and is associated with CRC recurrence [102].

CpG island methylator phenotype (CIMP). In fetal development, human body acquires DNA reading instructions on how organs are to be formed and preserved [77]. Epigenetics is the reading instruction on top (epi-) of DNA [77]. In order to ensure common functions and homeostasis, genes are turned on/off/up/down as reaction to inputs from epigenetics [77]. Epigenetics controls the expression of genes by attaching or removing chemical groups to DNA, chromatin and histones, in addition to modifying DNA structure and accessibility inside cell nuclei [77]. Such chemical groups are neither part of DNA, nor sequence modifiers [77]. They are mostly methyl-, phosphor-, and acetyl-groups [77].

DNA methylation, regulated enzymatically (DNA-methyltransferases - DNMT), is common throughout the genome [77]. Methylation plays an important role both in keeping genome stability and ensuring regulation of transcription [77]. Different methylation patterns are responsible for creation of cell lineages and cell differentiation [77]. Acting as an on/off switch, a methyl group which is added to cytosines in the promotor region can silence (suppress) a gene [77]. The methyl groups shut down transcription by preventing transcription factors from binding to the promotor region [77].

Unlike DNA, which is stably inherited from parents, gametic DNA methylation pattern is altered in early fetal life [103]. At embryo implantation stage, de novo DNA methylation takes place in almost all genome [103]. Nevertheless, a large group of promoter sequences (CpG islands) is recognized and protected from methylation: they predominantly represent house-keeping genes [77]. In the course of cell lineage differentiation and organogenesis, methylation pattern undergoes changes [104]. Some genes keep silenced by methylation and other organ-specific genes are demethylated, thereby acting as epigenetic template for tissue-specific gene expression [104].

In many cancer types, hypermethylation of CpG islands within promoter regions of genes (site-specific methylation) leads to blockage of transcription and reduction in tumour-suppressor gene activity, including DNA repair mechanisms, while global hypomethylation in other parts of genome is associated with genomic instability, chromosomal aberrations, overexpression of oncogenes and growth factors [105].

Altered methylation landscape is primarily confined to cancerous tissue and is difficult to be detected in blood, due to low yield of epigenetic material in samples and suboptimal sampling and purification methods [105].

In cancer, gene silencing by hypermethylation occurs approximately 10 times more frequently than by mutations [106]. In contrast to the 3 to 6 driver mutations and less than 100 passenger mutations usually found in sporadic CRC, 600 to 800 genes are transcriptionally silenced by hypermethylation of CpG islands in promotor regions, also known as site-specific DNA hypermethylation [106].

A CRC phenotype called CpG island methylator phenotype is marked by significant site-specific CpG-island hypermethylation of tumour suppressor genes, MSI, BRAF mutations and associated to right-sided CRC, mostly in elderly women [107]. CpG island methylator phenotype tumours often lack KRAS and P53 mutations [107]. They have mucinous histologic features (signet ring cells) and are associated with advanced T stage [107].

7. Management of Locally Advanced Rectal Cancer (LARC)

In the previous sections we analyzed epidemiological aspects, in addition to histopathological/immunohistochemical and genetic topics of CRC. As these three environments are concerned, large overlaps between colonic features and rectal cancer's ones make it difficult (if not impossible) to consider them as different neoplastic diseases.

Such statement turns out untrue when we consider the management of colonic and rectal cancer. As a matter of fact, the two types of cancers require different treatment approaches.

An introduction about the treatment of rectal cancer, mainly focusing on preoperative radiotherapy, allows a more thorough understanding of both study design and its results.

Rectum lower limit is commonly defined by anorectal ring, an anatomic landmark which is palpable at physical examination or radiographically visible as upper border of anal sphincter and puborectalis muscles [108,109]. Rectum upper limit has been differently defined as splaying of the teniae coli, sacral promontory, proximal valve of Houston, or level of peritoneal reflection [108,109].

A recent consensus conference defined the point of sigmoid take-off (i.e., the junction of the sigmoid mesocolon and mesorectum) as the upper limit of the rectum, according to what had been observed on cross-sectional imaging [110]. Given an imperfect correlation among these landmarks and inconsistent presence of all 3 valves of Houston, in a clinical perspective, rectum upper limit can be somewhat elusive [108,109]. All in all, location of rectal cancer is most commonly given by the distance from its distal margin to the anal verge and it is defined as the beginning of the hair-bearing skin [108,109]. Tumours having 15 cm distal extension from anal margin (as measured by rigid sigmoidoscopy) are classified as rectal ones, while more proximal tumours as colonic ones [108,109]. Cancers are called "low" (up to 5 cm), "middle" (from >5 to 10 cm) or "high" (from >10 up to 15 cm), although rectum total length can vary by body habitus and sex [111].

7.1 Rectal cancer preoperative radiotherapy

Patients with early (stage I) rectal cancer receive radical surgery (local excision, low anterior resection with total mesorectum excision) without any preoperative chemotherapy and/or radiotherapy. On the contrary, given the complexity and variability

of clinical presentation, which is strictly related to site (s), number of metastases and tumour burden, patients affected by metastatic rectal cancer (stage IV) cannot be reduced to standard of care algorithms (Table 1, Table 2).

Table 1. American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017

T Primary Tumour
TX Primary tumour cannot be assessed
T0 No evidence of primary tumour
Tis Carcinoma in situ: intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae)
T1 Tumour invades the submucosa (through the muscularis mucosa but not into the muscularis propria)
T2 Tumour invades the muscularis propria
T3 Tumour invades through the muscularis propria into pericolorectal tissues
T4 Tumour invades* the visceral peritoneum or invades or adheres** to adjacent organ or structure
T4a Tumour invades* through the visceral peritoneum (including gross perforation of the bowel through tumour and continuous invasion of tumour through areas of inflammation to the surface of the visceral peritoneum)
T4b Tumour directly invades* or adheres** to adjacent organs or structures
N Regional Lymph Nodes
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 One to three regional lymph nodes are positive (tumour in lymph nodes measuring ≥0.2 mm), or any number of tumour deposits are present and all identifiable lymph nodes are negative
N1a One regional lymph node is positive
N1b Two or three regional lymph nodes are positive
N1c No regional lymph nodes are positive, but there are tumour deposits in the subserosa, mesentery, or nonperitonealized pericolic, or perirectal/mesorectal tissues
N2 Four or more regional lymph nodes are positive
N2a Four to six regional lymph nodes are positive
N2b Seven or more regional lymph nodes are positive

M Distant Metastasis
M0 No distant metastasis by imaging, etc.; no evidence of tumour in distant sites or organs. (This category is not assigned by pathologists)
M1 Metastasis to one or more distant sites or organs or peritoneal metastasis is identified
M1a Metastasis to one site or organ is identified without peritoneal metastasis
M1b Metastasis to two or more sites or organs is identified without peritoneal metastasis
M1c Metastasis to the peritoneal surface is identified alone or with other site or organ metastases

Table 2. American Joint Committee on Cancer (AJCC) TNM Staging Classification for Rectal Cancer 8th ed., 2017 - Prognostic Groups

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

According to evidence-based guidelines, neoadjuvant radiotherapy is highly recommended to patients with clinical stage II-III rectal cancer [111]. Multiple prospective trials and meta-analyses have proved that neoadjuvant radiotherapy reduces risk of local recurrence, even in the era of total mesorectal excision (TME) [112-119].

Despite strong evidence supporting neoadjuvant radiotherapy approach in patients with stage II-III rectal cancer, a patient subset may be at low risk for locoregional recurrence, due to proximal tumour location and circumferential resection margin, which magnetic resonance imaging (MRI) defines as “safe” [116,120]. Based on such limited evidence, a conditional recommendation might suggest to omit neoadjuvant radiotherapy in favor of upfront surgery for patients in clinical stage IIA (cT3a/b N0), whenever cancer is located >10 cm from anal verge or in presence of ≥2 mm predicted circumferential resection margin and in the absence of extramural vascular invasion, as prescribed by MRI with rectal cancer protocol [121].

Long-course chemoradiotherapy (LCCRT), which represents the most common neoadjuvant regimen followed in USA, was analyzed by German Rectal Cancer trial (CAO/ARO/AIO-94), which randomly assigned patients with clinical stage II/III rectal cancer (n=823) to either preoperative LCCRT or postoperative one [114]. Chemoradiotherapy consisted of 5,040 cGy in 28 fractions, with concurrent infusional 5-fluorouracil (5FU) [114]. Standard TME was carried out and all patients underwent an additional 4 cycles adjuvant 5FU-based chemotherapy [114]. Local recurrence rate was lower in preoperative treatment group (6% versus 13%, p = 0.006) and such benefit endured at long-term (10-year) follow-up (7.1% versus 10%, p = 0.04) [114]. Meanwhile, no significant difference between the groups was recorded as concerned 10-year overall survival (OS), disease-free survival (DFS) or rate of distant metastasis [114]. When compared to postoperative therapy, preoperative LCCRT led to significantly less severe toxicity (grade 3 or 4), acute (27% versus 40%; p = 0.001) and long-term (14% versus 24%; p = 0.01) ones [114]. The most common acute toxicities related to preoperative therapy were diarrhea, hematologic and dermatologic damages, while the most common long-term harms included chronic diarrhea, bowel obstruction, anastomotic stricture, bladder and sexual dysfunctions [122]. Though meta-analyses have not yet stated that neoadjuvant LCCRT can lead to statistically significant differences in the rate of sphincter preservation, chemoradiotherapy may ease sphincter preservation by reducing tumour volume in deep pelvis [123].

Based on such findings, patients undergoing neoadjuvant conventionally fractionated radiotherapy are recommended 5,000 to 5,040 cGy in 25 to 28 fractions with concurrent chemotherapy [121].

Swedish rectal trial and Dutch rectal study, in additions to researches comparing neoadjuvant short-course radiotherapy (SCRT) with LCCRT, established 2,500 cGy in 5

fractions, without concurrent chemotherapy as standard of care for patients undergoing neoadjuvant SCRT [124,125].

Swedish Rectal Cancer trial randomly assigned 1,168 patients to SCRT followed by surgery versus surgery alone [124]. SCRT arm was associated with reduced local recurrence (9% versus 26%, $p < 0.001$) and prolonged survival (5-year OS, 38% versus 30%; $p = 0.008$) with a 13-year median follow-up [112,124].

Dutch TME trial randomly assigned 1,861 patients to SCRT, followed by TME surgery in comparison to TME surgery alone [125]. Even in a setting of standardized TME surgery, neoadjuvant SCRT significantly reduced local recurrence [125]. At 10 years, exclusive TME compared to SCRT and TME ($p < 0.001$) recorded an 11% local recurrence rate and a 5% one respectively, although no benefit was recorded in OS (49% after TME versus 48% after SCRT and TME, $p = 0.86$) [116]. Radiotherapy benefit proved to reach the highest level in tumours with nodal involvement located 5-10 cm from anal verge, with negative resection margins [116]. Neoadjuvant SCRT did not offset risk for local failure in low rectal tumours with positive resection margins [116]. SCRT addiction to TME led to higher levels of long-term toxicity [126]. In particular, higher fecal incontinence (62% versus 38%, $p < 0.001$), increased pad use (56% versus 33%, $p < 0.001$) and mucus leakage (27% versus 15%, $p = 0.005$) were reported in patients who had received radiation [126]. Irradiated males also reported more frequent sexual (erectile) problems [126,127].

A Cochrane database meta-analysis highlighted that patients undergoing SCRT followed by surgery were associated to lower local recurrence rates, if compared with patients who underwent exclusive surgery but they did not significantly increase sphincter-preservation rates or perioperative complication ones [117].

7.2 Long-course chemoradiotherapy versus Short-course radiotherapy

Polish trial (316 patients) and Trans-Tasman Radiation Oncology Group trial (TROG) 01.04 (326 patients) represent two randomized trials comparing neoadjuvant SCRT with LCCRT, which reported long-term oncologic outcomes [6,129]. The local recurrence, 5-year distant metastasis and OS rates did not differ significantly between either trial's arms [6,129]. Significantly lower acute toxicity was related to SCRT rather than to LCCRT (3% versus 18%, $p < 0.001$ for Polish trial; 1.9% versus 28%, $p < 0.001$ for TROG one), although rates of high-grade late toxicity did not significantly differ in either trials [6,128,129]. It is very important to underline that LCCRT turned out as

considerably more effective than SCRT in favouring pathologic downstaging and tumour regression. Although pooled analysis showed no discrepancy in the rates of sphincter preservation or R0 resection, pathological complete response (pCR) recorded a 16% rate following LCCRT, in comparison to a 1% value after SCRT ($p < 0.001$) [129,130].

The latest meta-analysis by Yu *et al* aimed at comparing LCCRT's and SCRT's outcomes and identify the most appropriate approach for rectal cancer preoperative treatment [131]. Both regimens proved effective in the preoperative treatment of LARC, taking into account pCR, tumour downstaging, local recurrence, distant metastases, mortality and serious late toxicity [131]. Furthermore, SCRT group without chemotherapy recorded a worse pCR rate compared to that of LCCRT [131]. This could explain the benefit of chemotherapy in preoperative treatment of RC. Given the significant heterogeneity within meta-analysis, a subgroup analysis was carried out, based on study types and intervention with chemotherapy [131]. Results underlined that LCCRT group showed a higher pCR prevalence and tumour downstaging in randomized control trial (RCT) subgroup [131]. Furthermore, LCCRT group also recorded a higher pCR prevalence compared with SCRT group without chemotherapy, suggesting that pCR may correlate with chemotherapy [131].

Stockholm III trial provided data regarding 385 patients with rectal cancer [132]. They were randomly assigned to SCRT with immediate surgery (SCRT), SCRT with delayed surgery at 4-8 weeks (SCRT-delay), or LCCRT with delayed surgery at 4-8 weeks (LCCRT-delay) [132]. Data concerned an additional 455 patients randomly assigned to SCRT or SCRT delay [132]. After a median follow-up of 5.2 years, overall local recurrence rate did not statistically differ among the 3 groups [132]. Although no discrepancy turned out in postoperative complication rates among patients in the 3-arm randomization, pooled analysis comparing patients treated by SCRT versus SCRT-delay showed a significantly lower risk of postoperative complications after SCRT-delay (41% versus 53%; OR = 0.61; 95% CI, 0.45–0.83; p = 0·001) and a higher rate of pCR (11.8% versus 1.7%, p = 0.001) [132-134]. These findings suggested that SCRT-delay may ease tumour regression, an advantage which is more commonly attributed to LCCRT [132-134].

In a perspective which considers efficiency and safety, a recent meta-analysis by Qiaoli *et al* explored SCRT with delayed surgery versus preoperative LCCRT [135]. It revealed no statistically significant difference in terms of OS, DFS, pCR, treatment-related grade

3/4 toxicity, postoperative complications (PCs), local recurrence (LR) or distant metastasis [135]. The two subgroups were then divided, in case any additional chemotherapy was available in SCRT arm [135]. The subgroup analysis showed no significant difference, as concerned DFS, distant metastasis, PCs or LR [135]. When compared with LCCRT group, SCRT group without adjuvant chemotherapy did not only record a lower treatment-related grade 3/4 toxicity (RR = 0.19, 95% CI 0.08–0.48, $P < 0.01$) but it also significantly showed lower levels of OS and pCR (HR = 2.05, 95% CI 1.13–3.72, P = 0.02; RR = 0.42, 95% CI 0.30–0.60, $P < 0.01$, respectively) [135].

To our surprise, pCR showed an increasing trend in SCRT with adjuvant chemotherapy group, although such discrepancy was not significant (RR = 1.37, 95% CI 0.90–2.09, P = 0.14) [135].

To date, conventionally fractionated long-course chemoradiation or short-course radiotherapy are equally recommended for patients requiring neoadjuvant therapy, due to high-quality evidence that both approaches can improve local control. Randomized studies suggest similar efficacy and patient reported QoL outcomes in either treatment.

8. Materials and Methods

8.1. Patients Cohort

A retrospective study was performed on surgical resection specimens of 95 patients with rectal adenocarcinoma, clinical stages II/III, treated with preoperative radiotherapy and radical surgery between 2000 and 2017 at the Azienda Unità Sanitaria Locale-IRCCS of Reggio Emilia, Italy.

Patient population was divided into two groups according to the preoperative radiation protocol: SCRT vs LCCRT.

The first group consisted of 45 patients (27 males and 18 females), having mean age 75.2 (range: 46-90 years), treated with SCRT [duration: 5 consecutive days with a total dose of 25 gray (Gy) in 5 fractions of 5 Gy daily] with immediate surgery performed within 7–10 days after the end of radiation.

The second group of 50 patients (25 males and 25 females), having mean age 62.4 (range: 38-79 years), included unresectable or very low-seated neoplasms. The latter group received LCCRT [duration of radiotherapy: 4–6 weeks, with a total dose of 45–50 Gy fractioned in single doses of 1.8–2 Gy daily] with surgery performed 4–6 weeks later.

In both protocols computerized tomography (CT) scan (with intravenous contrast medium administration) of lower abdomen and pelvis was performed. CT scan images were transferred to the pretreatment planning system for contouring the target volume and organs at risk. The gross total volume (GTV) was contoured based on clinical data, endoscopic ultrasound (EUS) and MRI. The clinical target volume (CTV) included at least a 3 cm craniocaudal margin to the GTV plus mesorectum, presacral and internal iliac lymph nodes. The external iliac nodes were included for T4 tumours involving anterior structures.

LCCRT patients were subsequently treated with chemotherapy.

8.2. Morphological examination

All hematoxylin and eosin-stained slides were reviewed independently by two expert pathologists. None of the examined cancers displayed neuroendocrine differentiation before radiotherapy. Non-neoplastic mucosa and tumour mucosa sections were assessed to detect radiation-induced changes and endocrine cells presence. Representative tumour sections were selected for grading of tumour regression according to Dworak *et*

al [136] system in 5 points as follows: grade 0 - no regression; grade 1 - dominant tumour mass with obvious fibrosis and/or vasculopathy; grade 2 - dominantly fibrotic changes with few tumour cells or groups; grade 3 - very few tumour cells in fibrotic tissue with or without mucous substance; grade 4 - no tumour cells, only fibrotic mass (total tumour regression).

The degree of radiation damage was assessed in the non-neoplastic mucosa, including the surgical resection margins. The following histological parameters were evaluated: i) inflammation in the lamina propria; ii) architectural crypt distortion; iii) nuclear and cytoplasmic atypia of glandular epithelium; iv) apoptotic bodies. All these features were categorized as being present or absent and, if present, semi-quantitatively graded as mild, moderate and severe.

8.3. Immunohistochemical analysis

In 25 SCRT and 25 LCCRT cases, the presence of endocrine features was assessed morphologically and with chromogranin A (LK 2H10, monoclonal antibody, Ventana, Oro Valley, AZ, USA) and synaptophysin (SP11, monoclonal antibody, Ventana, Oro Valley, AZ, USA) immunostainings in both neoplastic and radiation-damaged non-neoplastic mucosae.

The endocrine differentiation was scored as follows: i) absent; ii) isolated endocrine cells; iii) endocrine cells micronests. Endocrine micronests were defined as 5–15 cells clusters.

In a subset of 22 SCRT patients (the same subset in which samples were eligible for genomic DNA analysis), p53 (DO-7 monoclonal antibody, Ventana, Oro Valley, AZ, USA) immunohistochemical expression was evaluated in both neoplastic and radiation-damaged non-neoplastic mucosae.

8.4. Next Generation Sequencing (NGS) mutational analysis

Genomic DNA, from both neoplastic and “dysplastic-like” mucosa samples of a subset of 24 SCRT patients was isolated by Maxwell DNA FFPE Kit (Promega, Madison, WI, USA), according to the manufacturer instructions. DNA concentration was determined using Qubit dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Twenty-two samples were eligible for sequencing analysis. For the NGS analysis, DNA libraries were prepared using Myriapod NGS-IL 56G Onco Panel for Illumina (Diatech Pharmacogenetics, Jesi, Italy), that allows the identification of main mutations in 56

oncogenes, following the manufacturer instructions. Libraries quality and quantity were assessed by Agilent Bioanalyzer High Sensitivity kit (Agilent Technologies, Santa Clara, CA, USA) and Qubit dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA) respectively. Sequencing run on Illumina MiSeq V2 (2x151) cartridge (Illumina, San Diego, CA, USA). Sequencing data analysis was conducted by Myriapod NGS Analysis software (v 4.0.2) and further analysis were performed using R software (v 3.5.1).

8.5. Statistical analysis

All statistical analysis in this study were elaborated using R software (v 3.5.1).

Stastical comparisons of Clinicopathological and Endocrine features between SCRT and LCCRT groups were performed applying *Fisher test* for categorical variables and *Kruskal Wallis test* for continuous variables.

Waterfall plot of genetic alterations was created using R "GenVisR" package including both germinal and somatic variants that passed quality filters applied by default by Myriapod NGS Analysis software.

Differences were considered statistically significant with a p value <0.05.

9. Results

The clinicopathological features of SCRT and LCCRT cases are summarized in Table 3.

Table 3. Clinicopathological features of SCRT and LCCRT cases.

	SCRT	**%**	**LCCRT**	**%**	**P value**
	45	**47.4%**	**50**	**52.6%**	
Age	75.2 years (46-90)		62.4 years (38-79)		<0.001
Sex					0.410
Female	18	18.9%	25	26.3%	
Male	27	28.4%	25	26.3%	
Tumour regression system by Dworak [16]					<0.001
Grade 0	31	32.6%	12	12.5%	
Grade 1	14	14.7%	15	15.8%	
Grade 2	0	0.0%	20	21.1%	
Grade 3	0	0.0%	2	2.1%	
Grade 4	0	0.0%	1	1.1%	
Inflammation					<0.001
absent	4	4.2%	35	36.7%	
mild	4	4.2%	13	13.7%	
moderate	23	24.2%	1	1.1%	
severe	14	14.7%	1	1.1%	
Architectural crypt distortion					<0.001
absent	4	4.2%	47	49.4%	
mild	4	4.2%	2	2.1%	
moderate	19	20.0%	1	1.1%	
severe	18	18.9%	0	0.0%	
Nuclear and cytoplasmic atypia of glandular epithelium					<0.001
absent	4	4.2%	49	51.5%	
mild	3	3.2%	1	1.1%	
moderate	20	21.1%	0	0.0%	
severe	18	18.9%	0	0.0%	
Apoptotic bodies					<0.001
absent	5	5.3%	49	51.5%	
present	40	42.1%	1	1.1%	

SCRT: short-course radiotherapy; LCCRT: long-course chemoradiotherapy

9.1. Tumour regression

According to the tumour regression system by Dworak *et al* [136], tumours were classified as not regressed (Grade 0) or with regression from Grade 1 to Grade 4. In the SCRT group, 31 cases were classified as not regressed (Grade 0) and 14 cases as Grade 1. In the LCCRT group the tumours were classified as follows: 12 cases Grade 0; 15 cases Grade 1; 20 cases Grade 2; 2 cases Grade 3 and 1 case Grade 4.

9.2. Radiation-induced morphological features

The radiation-induced morphological features were noted in non-neoplastic mucosa samples taken within the irradiated volume. They were identified in the resection margins, if within the irradiated volume. Two types of parameters were analyzed: i) inflammatory component and ii) glandular ("dysplastic-like") abnormalities.

9.3. Inflammatory component

In SCRT cases, the non-neoplastic mucosa within the irradiated volume showed a moderate to marked inflammation within the lamina propria (Figure 9). The inflammation consisted of histiocytes, lymphocytes, plasma cells and typically numerous eosinophils. The eosinophils were identified in the lamina propria either scattered or in small aggregates and within the glandular epithelium (Figure 10). In LCCRT samples the inflammation went from absent to a mild chronic inflammatory infiltrate. Eosinophils were rare.

Figure 9. Low power view of radiation-damaged non-neoplastic mucosa in SCRT sample: the lamina propria looks expanded by inflammatory cells and crypts (yellow arrows) are decreased (HE 40X). [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]

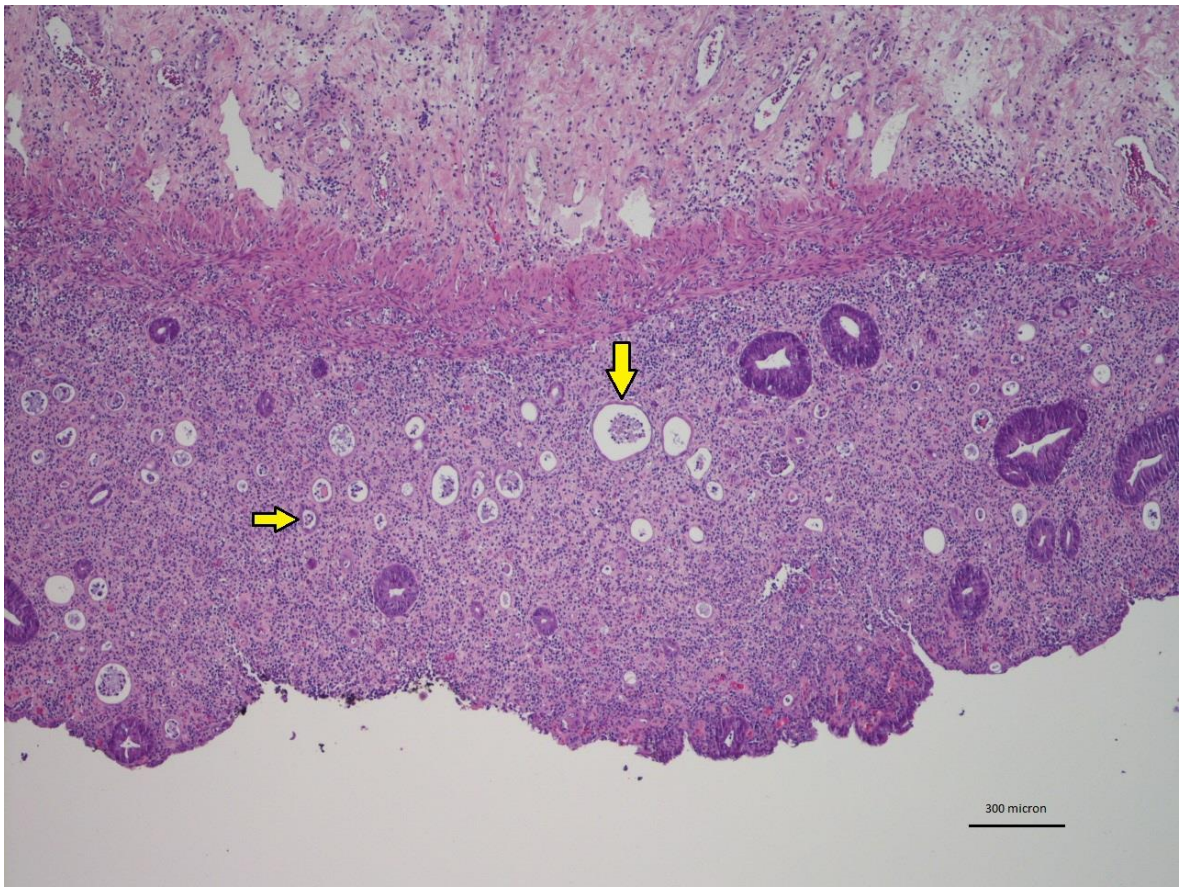
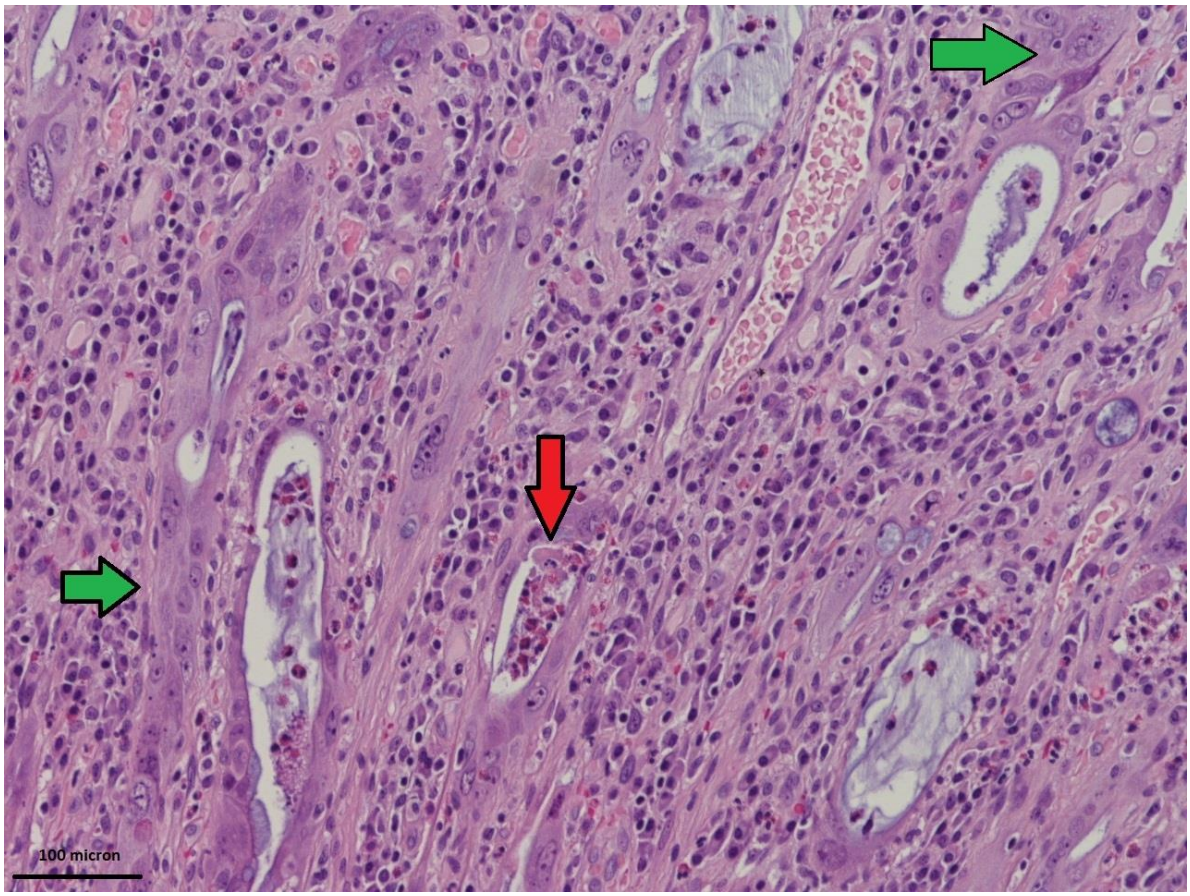


Figure 10. High power view of radiation-damaged non-neoplastic mucosa in SCRT sample: distorted glands lined by atypical epithelium (green arrows) and eosinophils aggregates (red arrow) within glandular epithelium (HE 200X). [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



9.4. "Dysplastic-like" features

In SCRT cases, there was a moderate to marked degree of glandular disarray/distortion as well as nuclear and cytoplasmic atypia. The crypts were decreased in number, dilated or with slit-like lumen (Figure 11). The crypt epithelium was either flattened or pseudostratified showing a variable degree of nuclear pleomorphism (Figure 12 *left/right*). The cytoplasm of the crypt epithelium was eosinophilic or vacuolated. Apoptotic bodies were identified. All these features were named "dysplastic-like" for simplicity.

In contrast to the short-course group, in LCCRT cases the "dysplastic-like" features were either absent or occasionally identified.

Figure 11. "Dysplastic-like" features in radiation-damaged non-neoplastic mucosa in SCRT sample: glands with slit-like lumen and flattened epithelium close to glands with pseudostratified epithelium (HE 200x). [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]

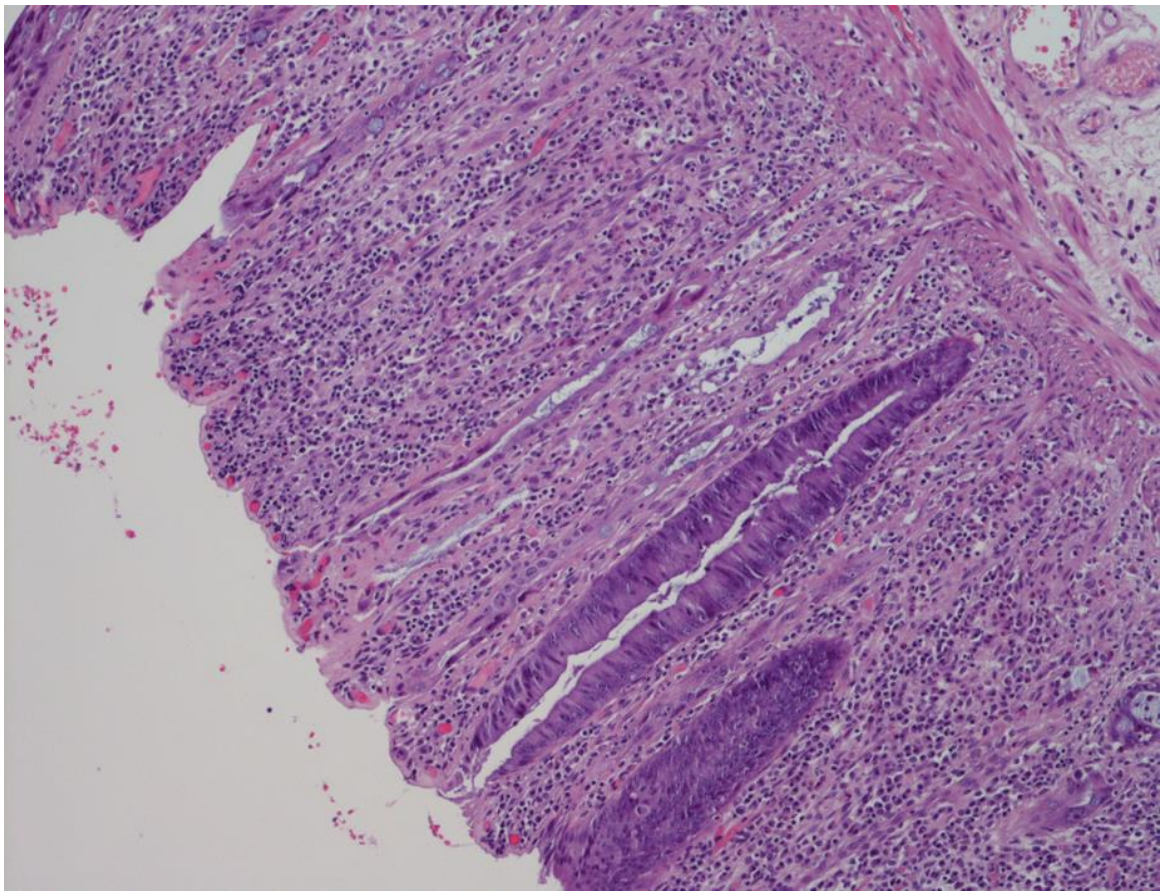
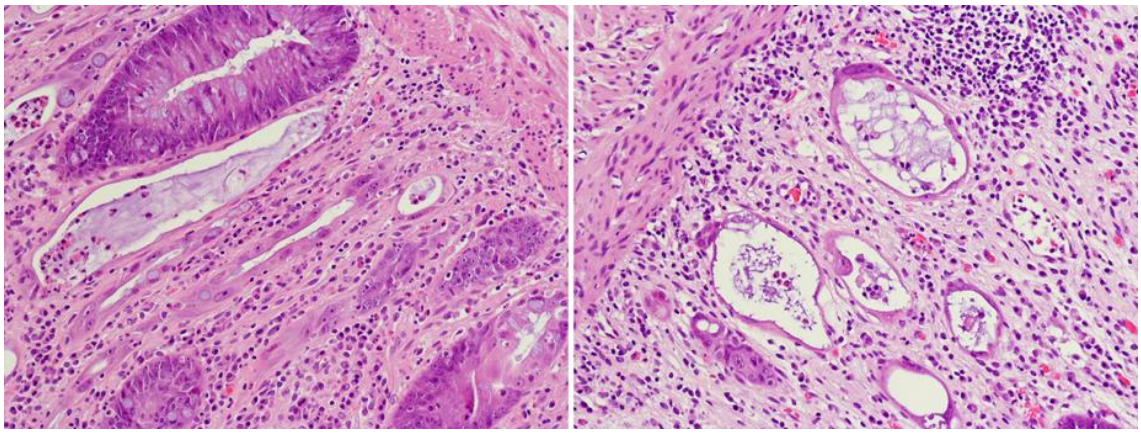


Figure 12. *left/right* "Dysplastic-like" features in radiation-damaged non-neoplastic mucosa in SCRT sample: dilated glands with atypical epithelium are present (HE 200x). [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



9.5. Endocrine features

The presence of endocrine cells showed differences according to the type of protocol (Table 4).

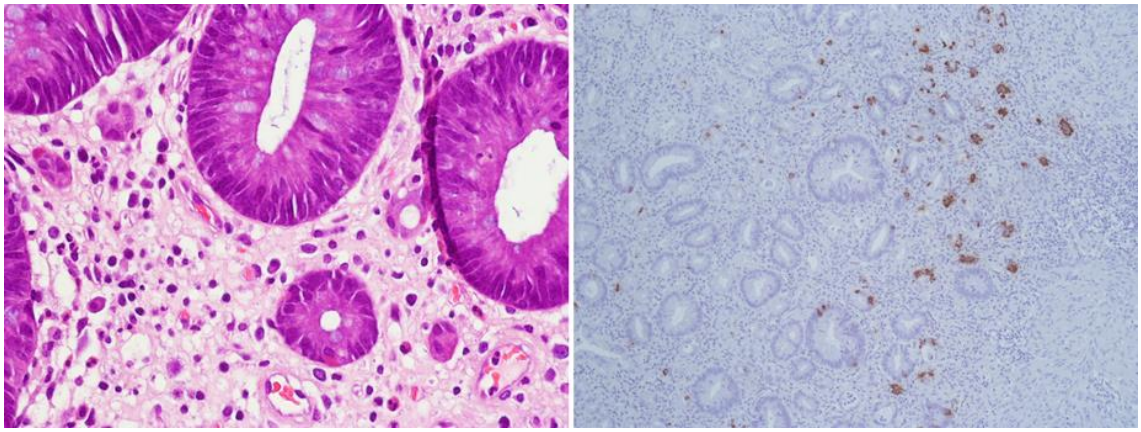
Table 4. Endocrine features in SCRT and LCCRT cases.

	SCRT		LCCRT		P value
	N°	%	N°	%	
	25	50.0%	25	50.0%	
Endocrine differentiation in radiation-damaged mucosae					<0.001
absent	1	2.0%	20	40.0%	
isolated endocrine cells	5	10.0%	5	10.0%	
endocrine cells micronests	19	38.0%	0	0.0%	
Endocrine differentiation in tumour					0.490
absent	23	46.0%	25	50.0%	
isolated endocrine cells	1	2.0%	0	0.0%	
endocrine cells micronests	1	2.0%	0	0.0%	

SCRT: short-course radiotherapy; LCCRT: long-course chemoradiotherapy

In SCRT cases, the radiation-damaged mucosa showed an increase in endocrine cells (Figure 13 *left/right*) either with isolated cells (5 cases) or micronests (19 cases), absence of endocrine cells was seen in one case. In the LCCRT group, the non-neoplastic mucosa within the irradiated volume showed mainly absence of endocrine cells (20 cases) and, more rarely, isolated cells (5 cases). No relevant differences were seen in terms of endocrine differentiation in tumour samples of both protocols.

Figure 13. *left* Endocrine cells in radiation-damaged non-neoplastic mucosa in SCRT sample (HE 200x); *right* Chromogranin immunostaining highlighting endocrine cells in radiation-damaged non-neoplastic mucosa in SCRT sample. [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



9.6. p53 immunohistochemical results

In a subset of 22 SCRT cases, different p53 staining patterns were identified in tumours (Table 3).

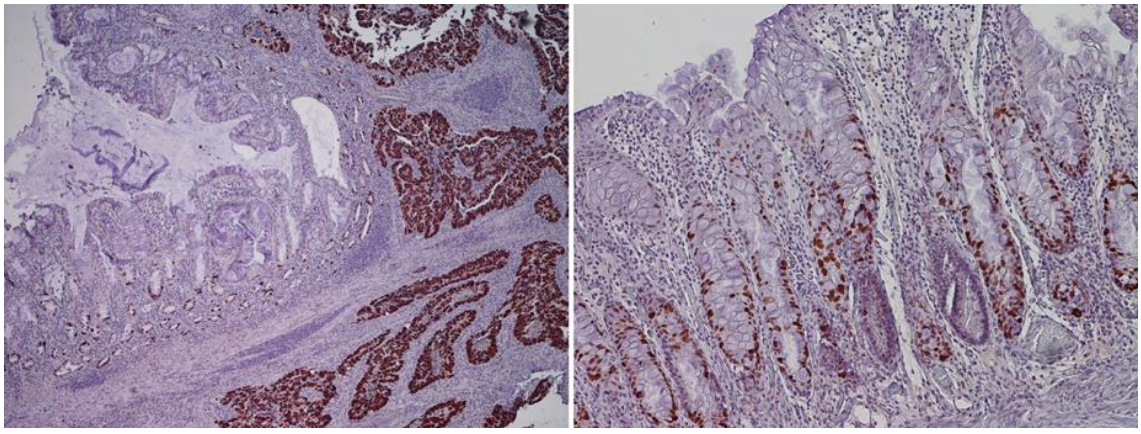
Table 5. Comparative data between p53 phenotype and TP53 genotype in SCRT tumour samples.

p53 IHC	TP53 NGS		Total of cases
	MUT	WT	
Negative-pattern	2	3	5
Positive-pattern	9	6	15
Reactive-pattern	0	2	2
Total of cases	**11**	**11**	**22**

IHC: immunohistochemistry; NGS: next generation sequencing; MUT: mutated; WT: wild-type

A strong and diffuse p53 expression ("positive-pattern") was present in 15/22 tumours (Figure 14 *left*) a complete lack of expression ("negative-pattern") was seen in 5/22 tumours; scattered p53-positive cells ("reactive-pattern") were present in 2/22 tumours. In all samples examined, the mucosa with acute radiation-damage (data not shown in Table 5) showed a positive p53 staining limited to the deep portion of the glandular epithelium which represents the proliferative compartment of the glands (Figure 14 *right*).

Figure 14. *left* "Positive-pattern" of p53 in SCRT tumour sample (right). Few scattered p53-positive cells in mucosa with acute radiation-damage (left) (p53 immunostaining); *right* Scattered p53-positive cells in the deep portion of glandular epithelium of radiation-damaged non-neoplastic mucosa in SCRT (p53 immunostaining). [Dr. Magda Zanelli and Dr. Loredana De Marco; Pathology Unit-Azienda Unità Sanitaria Locale – IRCCS di Reggio Emilia, Italy]



9.7. Genetic alterations in tumours and in tissue samples with "dysplastic-like" features

We evaluated quality of 48 tissue DNA from both tumour and “dysplastic-like” mucosa of 24 SCRT patients included in the study. Only in 22 patients we obtained, from both components, DNA eligible for NGS analysis. On these samples, we performed a deep sequencing analysis on a commercial panel of 56 genes frequently mutated in cancer, detecting 958 alterations (Figure 15 *A*). Subsequently, the analysis was restricted to coding regions variants, excluding intronic, 50-30 UTR and downstream gene variants. 266 mutations were found, of which 146 (54.9%) were synonymous, 103 (38.7%) missense and a small percentage was composed by stop gained and frameshift alterations (4.1% and 2.3%, respectively) (Figure 15 *B*). Based on literature, Exome Aggregation Consortium (ExAc) frequency and variant frequency 230/266 alterations (86.5%) were classified as germinal and 36/266 (13.5%) as somatic. In each patient, “dysplastic-like” mucosa and tumour, shared the same germline alterations confirming the constitutiveness of these variants and the validity of our analysis (Table 6). By contrast, somatic mutations were present only in tumours, suggesting that “dysplastic-like” tissues are not genetically transformed (Figure 16). Between tumour-associated somatic mutations 52.8% were missense, 30.6% were stop gained and 16.6% were frameshift (Figure 15 *C*).

Somatic mutations were detected in 7/56 genes and gene alterations frequencies were in line with The Cancer Genome Atlas (TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga)) data on CRC. The most frequently mutated genes were APC and TP53 detected in 47.8% of patients. 66.7% of the described somatic alterations were annotated in these genes, mutations in APC were in particular stop gained and frameshift variants while TP53 presented a majority of missense mutations. Moreover, 26.1% of analyzed tumours presented missense mutations in KRAS (6/22) and 13% in PIK3CA. Finally, only one tumour presented a missense mutation in FBXW7 and a stop mutation in SMAD4 and one had a missense mutation in EGFR (Figure 15 *D*,*E*).

Figure 15. Mutational Profile of 44 tumours and mucosa tissues from 22 PSRT patients: (**A**) Distribution of 958 genetic alterations according with position and functional effects; (**B**) Distribution of 266 coding region variants according with predicted functional effects; (**C**) Distribution of 36 tumour-associated somatic mutations according with predicted functional effects; (**D**) Frequency distribution of somatic mutated genes in analysed patients; (**E**) Frequency distribution of somatic variants with different functional effect in mutated genes.

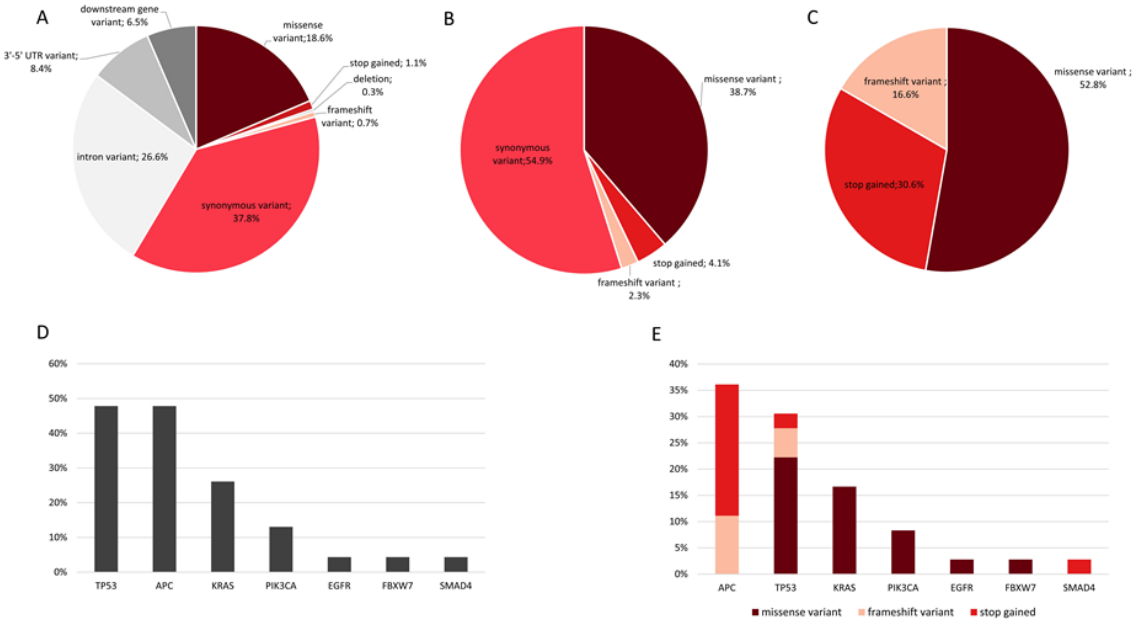


Table 6. List of genetic variants detected in tumour and normal tissue of each patient. For each variant coding effect and origin are reported.

	Tumour		Normal			
	Gene	**AA Change**	**Gene**	**AA Change**	**Coding Effect**	**Origin**
Patient 1	EGFR	p.Ala613Ala	EGFR	p.Ala613Ala	synonymous_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KIT	p.Met541Leu	KIT	p.Leu862Leu	missense_variant	germline
	KIT	p.Leu862Leu	KIT	p.Met541Leu	synonymous_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
Patient 2	APC	p.Ile1307Lys	APC	p.Ile1307Lys	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
Patient 4	EGFR	p.Gln787Gln	EGFR	p.Gln787Gln	synonymous_variant	germline
	FGFR3	p.Phe384Leu	FGFR3	p.Phe384Leu	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	MET	p.Ser178Ser	MET	p.Ser178Ser	synonymous_variant	germline
	PIK3CA	p.Ile391Met	PIK3CA	p.Ile391Met	missense_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	APC	p.Arg876*			stop_gained	somatic
	APC	p.Ile1307fs			frameshift_variant	somatic
	TP53	p.Arg273Cys			missense_variant	somatic
Patient 5	AKT1	p.Gln43His	AKT1	p.Gln43His	missense_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	TP53	p.Pro36Pro	TP53	p.Pro36Pro	synonymous_variant	germline
	TP53	p.Arg213Arg	TP53	p.Arg213Arg	synonymous_variant	germline

	FBXW7	p.Arg347Cys			missense_variant	somatic
	PIK3CA	p.Glu542Lys			missense_variant	somatic
	SMAD4	p.Gln245*			stop_gained	somatic
	TP53	p.Pro152Leu			missense_variant	somatic
Patient 6	EGFR	p.Gln787Gln	EGFR	p.Gln787Gln	synonymous_variant	germline
	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	MET	p.Arg970Cys	MET	p.Arg970Cys	missense_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	APC	p.Glu1379*			stop_gained	somatic
	EGFR	p.Asp855Gly			missense_variant	somatic
	KRAS	p.Gly12Val			missense_variant	somatic
	PIK3CA	p.Glu542Val			missense_variant	somatic
	TP53	p.His214Arg			missense_variant	somatic
Patient 7	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	PIK3CA	p.Ile391Met	PIK3CA	p.Ile391Met	missense_variant	germline
	APC	p.Gln1367*			stop_gained	somatic
	KRAS	p.Gly12Asp			missense_variant	somatic
	PIK3CA	p.Glu545Lys			missense_variant	somatic
	TP53	p.Gly293fs			frameshift_variant	somatic
Patient 8	ATM	p.Pro604Ser	ATM	p.Pro604Ser	missense_variant	germline
	KDR	p.Val297Ile	KDR	p.Val297Ile	missense_variant	germline
	KIT	p.Lys546Lys	KIT	p.Lys546Lys	synonymous_variant	germline
	MSH6	p.Gly1072Val	MSH6	p.Gly1072Val	missense_variant	germline
	APC	p.Thr1301fs			frameshift_variant	somatic
	TP53	p.Arg175His			missense_variant	somatic
Patient 9	ATM	p.Phe858Leu	ATM	p.Phe858Leu	missense_variant	germline
	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	FGFR3	p.Asn294Asn	FGFR3	p.Asn294Asn	synonymous_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	IDH1	p.Gly105Gly	IDH1	p.Gly105Gly	synonymous_variant	germline
	KDR	p.Cys482Arg	KDR	p.Cys482Arg	missense_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	PIK3CA	p.Ile391Met	PIK3CA	p.Ile391Met	missense_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	TP53	p.Arg213Arg	TP53	p.Arg213Arg	synonymous_variant	germline

	TSC2	p.Ser1557Ser	TSC2	p.Ser1557Ser	synonymous_variant	germline
	APC	p.Arg1114*			stop_gained	somatic
	KRAS	p.Gly12Val			missense_variant	somatic
Patient 10	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	TSC2	p.Ala1560Ala	TSC2	p.Ala1560Ala	synonymous_variant	germline
	KRAS	p.Gly12Ala			missense_variant	somatic
	TP53	p.Arg306*			stop_gained	somatic
Patient 12	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
Patient 13	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	PIK3CA	p.Ile391Met	PIK3CA	p.Ile391Met	missense_variant	germline
	APC	p.Glu1309fs			frameshift_variant	somatic
	TP53	p.Leu257Pro			missense_variant	somatic
Patient 14	EGFR	p.Gln787Gln	EGFR	p.Gln787Gln	synonymous_variant	germline
	ERBB2	p.Leu696Leu	ERBB2	p.Leu696Leu	synonymous_variant	germline
	FGFR3	p.Asn294Asn	FGFR3	p.Asn294Asn	synonymous_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KDR	p.Val297Ile	KDR	p.Val297Ile	missense_variant	germline
	APC	p.Arg1114*			stop_gained	somatic
Patient 15	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	KDR	p.Val297Ile	KDR	p.Val297Ile	missense_variant	germline
	MET	p.Ser178Ser	MET	p.Ser178Ser	synonymous_variant	germline
	MET	p.Asn375Ser	MET	p.Asn375Ser	missense_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	TP53	p.Val272Leu			missense_variant	somatic
Patient 16	FGFR3	p.Asn294Asn	FGFR3	p.Asn294Asn	synonymous_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	IDH1	p.Gly105Gly	IDH1	p.Gly105Gly	synonymous_variant	germline
	KDR	p.Val297Ile	KDR	p.Val297Ile	missense_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	MET	p.Arg970Cys	MET	p.Arg970Cys	missense_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
Patient 17	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	KIT	p.Lys546Lys	KIT	p.Lys546Lys	synonymous_variant	germline

	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	APC	p.Ser1356*			stop_gained	somatic
	TP53	p.Leu188fs			frameshift_variant	somatic
Patient 19	EGFR	p.Ala613Ala	EGFR	p.Ala613Ala	synonymous_variant	germline
	FLT3	p.Ile827Leu	FLT3	p.Ile827Leu	missense_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
Patient 20	ATM	p.Asp2661Asp	ATM	p.Asp2661Asp	synonymous_variant	germline
	IDH1	p.Gly105Gly	IDH1	p.Gly105Gly	synonymous_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	KIT	p.Met541Leu	KIT	p.Leu862Leu	missense_variant	germline
	KIT	p.Leu862Leu	KIT	p.Met541Leu	synonymous_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
Patient 21	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
	KIT	p.Leu862Leu	KIT	p.Leu862Leu	synonymous_variant	germline
	KDR	p.Val297Ile	KDR	p.Val297Ile	missense_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	APC	p.Gln1367*			stop_gained	somatic
Patient 22	EGFR	p.Ala613Ala	EGFR	p.Ala613Ala	synonymous_variant	germline
	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KRAS	p.Gly12Asp			missense_variant	somatic
Patient 23	HRAS	p.His27His	HRAS	p.His27His	synonymous_variant	germline
	KIT	p.Met541Leu	KIT	p.Met541Leu	missense_variant	germline
	KIT	p.Leu862Leu	KIT	p.Leu862Leu	synonymous_variant	germline
	RET	p.Ser904Ser	RET	p.Ser904Ser	synonymous_variant	germline
	APC	p.Arg876*			stop_gained	somatic
	APC	p.Gly1466fs			frameshift_variant	somatic
	KRAS	p.Gly13Asp			missense_variant	somatic
	TP53	p.Glu258Asp			missense_variant	somatic
Patient 24	IDH1	p.Gly105Gly	IDH1	p.Gly105Gly	synonymous_variant	germline
	KDR	p.Cys482Arg	KDR	p.Cys482Arg	missense_variant	germline
	KDR	p.Gln472His	KDR	p.Gln472His	missense_variant	germline
	KRAS	p.Asp173Asp	KRAS	p.Asp173Asp	synonymous_variant	germline
	MET	p.Ser178Ser	MET	p.Ser178Ser	synonymous_variant	germline
	MET	p.Asn375Ser	MET	p.Asn375Ser	missense_variant	germline

	PDGFRA	p.Val824Val	PDGFRA	p.Val824Val	synonymous_variant	germline
Patient 25	ERBB2	p.Ile655Val	ERBB2	p.Ile655Val	missense_variant	germline
	FGFR3	p.Phe384Leu	FGFR3	p.Phe384Leu	missense_variant	germline
	IDH1	p.Gly105Gly	IDH1	p.Gly105Gly	synonymous_variant	germline
	KIT	p.Lys546Lys	KIT	p.Lys546Lys	synonymous_variant	germline
	APC	p.Gln1291*			stop_gained	somatic
	TP53	p.Arg248Gln			missense_variant	somatic

9.8. Comparison between p53 phenotype and TP53 genotype in tumour and mucosa with "dysplastic-like" features

Mutant TP53 was associated with diffuse and intense p53 immunostaining ("positive-pattern") in 9/22 tumours. This "positive-pattern" was also present in 6/22 tumours with wild-type TP53. Of 5/22 completely p53-negative ("negative-pattern") tumours, 2 cases had a mutation of TP53 and 3 cases were wild-type TP53. Two tumours with wild-type TP53 showed only rare, scattered p53-positive cells ("reactive-pattern").

All 22 samples of mucosa with "dysplastic-like" features were TP53 wild-type and showed p53 immunostaining only in the deep, proliferative portion of the glandular epithelium.

10. Discussion

Preoperative or neoadjuvant radiotherapy is used increasingly in the management of clinical II/III stages rectal cancer patients.

It is essential for pathologists to be familiar with radiation-induced morphological modifications. Radiation-induced changes in the tumour are well described, particularly tumour down-staging as a consequence of long-term radiotherapy [136]. Less attention has been given to the non-neoplastic mucosa.

Chronic radiation colitis pattern (dilated capillaries within hyalinized lamina propria), identified months or years after radiotherapy, is well known by pathologists [137]. Acute radiation colitis histology is occasionally described [14].

Intrigued by the observation that SCRT-associated epithelial changes simulate dysplasia, we designed the current study comparing LCCRT cases with SCRT ones. The short time interval between the end of radiotherapy and surgery is the reason why in the short-term group we found acute radiation colitis features (i.e., acute inflammation rich in eosinophils, crypt distortion, epithelial atypia, apoptotic bodies). These changes were restricted to the mucosa included in the irradiated volume.

“Dysplastic-like” features in irradiated normal mucosa were observed only in SCRT specimens. In esophagus carcinoma Brien *et al* noted that radiation-induced atypia within benign glands mimics dysplasia or even residual carcinoma [138]. The expanding use of SCRT leads the pathologists to have to evaluate acute radiation colitis and its differential. The misinterpretation of acute radiation colitis as dysplasia is a significant diagnostic error. According to our experience as well as that of most of our colleagues, intraoperative pathological analysis of distal section margin related to patients undergoing preoperative SCRT and anterior resection of rectum for locally advanced medium/low rectal cancer has often led to misinterpretations. As a matter of fact, histological similarities between SCRT-induced dysplasia and rectal neoplasia often arouse misunderstanding or made it difficult to discriminate between the two types of mucosa.

When frozen sections are performed on resection margins, an erroneous diagnosis of dysplasia can cause patient overtreatment. As a consequence, intraoperative distal extension of rectal resection turned out compulsory although, on final histological examination, it proved unnecessary. Unfortunately, distal extension of resection increased risk of both short- and long-term functional surgical complications, Low

Anterior Resection Syndrome (LARS), in particular, and defined as a constellation of symptoms including incontinence, frequency, urgency, or feelings of incomplete emptying, which has a significant impact on quality of life [139].

CRC is the result of accumulation of multiple genetic and epigenetic aberrations [140]. CRC begins as a benign adenomatous intestinal polyp, evolving to adenoma with high grade dysplasia, invasive adenocarcinoma and metastatic disease [140]. According to the multistep genetic model by Fearon and Vogelstein, the APC (adenomatous polyposis) mutation is the first event transforming normal colorectal epithelium to adenoma [75]. APC inactivation is followed by oncogenic KRAS mutations in the adenomatous stage and eventually chromosome 18q deletion and inactivation of tumour-suppressor gene TP53 on chromosome 17p in the transition to malignancy.

In a subset of SCRT cases, we performed NGS analysis on both tumour and irradiated mucosa with “dysplastic-like” features. Somatic mutations were found only in tumour samples. The most frequently mutated genes were TP53 and APC, consistently with the literature data reporting APC as the most frequently found mutation in CRC followed by TP53. Somatic mutations were not identified in mucosa with acute radiation colitis changes, supporting the concept that tissues with features mimicking dysplasia were not genetically transformed.

Consistently with previous studies [14,15], p53 overexpression (positive-pattern) was found to closely correlate with TP53 mutation, as in most tumours with mutated TP53 (9/11; 81.8%) a diffuse and intense p53 staining was present. This positive pattern is generally considered indicative of a missense TP53 mutation. As expected, a complete absence of nuclear staining (negative-pattern) was identified in 2 TP53 mutated tumours (18.1%) presenting a stop gained or frameshift variant responsible for a protein loss of function [16,17]. Non-concordant data were obtained only in 6 of the investigated samples.

This is not surprising being already reported in literature that IHC and NGS may sometimes result in divergent conclusions. While a formal explanation for this it has not yet been provided, this discrepancy is likely associated with p53 alterations, like copy number variation (CNV), which cannot be evaluated by the employed NGS approach.

Interestingly neither the positive- or negative-patterns of p53 staining were seen in the mucosa with acute radiation damage. p53 labeling was restricted to the proliferative compartment of the glandular epithelium in the mucosa with “dysplastic-like” features,

in keeping with the physiologic activity of p53 protein. Accordingly, the mucosa with radiation-induced atypia was consistently TP53 wild-type.

An increase in endocrine cells was noted in the irradiated non-neoplastic mucosa in the SCRT group. In analogy to previous observations in esophageal adenocarcinoma after neoadjuvant treatment [141], we interpreted the endocrine cells as residual normal endocrine elements, appearing more conspicuous for the radiation-induced glandular damage causing “passive clustering” [142]. In support of this observation, no endocrine cells increase was observed in the non-neoplastic irradiated mucosa in LCCRT cases, in which acute radiation colitis features were characteristically absent. Unlike previous reports [141], which found the extent of endocrine differentiation within the tumour to be proportional to the degree of tumour regression, we did not observe increase in endocrine cells within the tumour in either group of patients.

11. Conclusions

The present data are a comprehensive morphological, and immunohistochemical description of radiation-induced abnormalities after preoperative radiotherapy for rectal cancer. NGS analysis supported the morphological concept that SCRT-induced “dysplastic-like” tissues are not genetically transformed.

Short-course radiotherapy may induce morphological features closely simulating dysplasia. The misinterpretation may lead to patient’s overtreatment. When facing rectal cancer specimens, pathologists need the complete patient’s clinical history and must ask if preoperative radiotherapy has been given. p53 immunostaining may be of help in problematic cases.

References

1. Camilla Mattiuzzi, Fabian Sanchis-Gomar, Giuseppe Lippi. *Concise update on colorectal cancer epidemiology.* Ann Transl Med. **2019** Nov;7(21):609. doi: 10.21037/atm.2019.07.91.

2. Matthew Fleming, Sreelakshmi Ravula, Sergei F Tatishchev, Hanlin L Wang. *Colorectal carcinoma: Pathologic aspects.* J Gastrointest Oncol. **2012** Sep;3(3):153-73. doi: 10.3978/j.issn.2078-6891.2012.030.

3. WHO Classification of Tumours Editorial Board. *Digestive System Tumours, 5th ed.*; IARC: Lyon, France, **2019**.

4. Jeroen C Hol, Stefan E van Oostendorp, Jurriaan B Tuynman, Colin Sietses. *Long-term oncological results after transanal total mesorectal excision for rectal carcinoma.* Tech Coloproctol. **2019** Sep;23(9):903-911. doi: 10.1007/s10151-019-02094-8.

5. Colorectal Cancer Collaborative Group. *Adjuvant radiotherapy for rectal cancer: a systematic overview of 8,507 patients from 22 randomised trials.* Lancet. **2001** Oct 20;358(9290):1291-304. doi: 10.1016/S0140-6736(01)06409-1.

6. Samuel Y Ngan, Bryan Burmeister, Richard J Fisher, Michael Solomon, David Goldstein, David Joseph, Stephen P Ackland, David Schache, Bev McClure, Sue-Anne McLachlan, Joseph McKendrick, Trevor Leong, Cris Hartopeanu, John Zalcberg, John Mackay. *Randomized trial of short-course radiotherapy versus long-course chemoradiation comparing rates of local recurrence in patients with T3 rectal cancer: Trans-Tasman Radiation Oncology Group trial 01.04.* J Clin Oncol. **2012** Nov 1;30(31):3827-33. doi: 10.1200/JCO.2012.42.9597.

7. Robert Siegel, Susen Burock, Klaus-Dieter Wernecke, Albrecht Kretzschmar, Manfred Dietel, Volker Loy, Stephan Koswig, Volker Budach, Peter M Schlag. *Preoperative short-course radiotherapy versus combined radiochemotherapy in locally advanced rectal cancer: a multi-centre prospectively randomised study of the Berlin Cancer Society.* BMC Cancer. **2009** Feb 6;9:50. doi: 10.1186/1471-2407-9-50.

8. Richard J Stephens, Lindsay C Thompson, Phil Quirke, Robert Steele, Robert Grieve, Jean Couture, Gareth O Griffiths, David Sebag-Montefiore. *Impact of short-course preoperative radiotherapy for rectal cancer on patients' quality of life: data from the Medical Research Council CR07/National Cancer Institute of Canada Clinical Trials Group C016 randomized clinical trial.* J Clin Oncol. **2010** Sep 20;28(27):4233-9. doi: 10.1200/JCO.2009.26.5264.

9. K Bujko, M P Nowacki, A Nasierowska-Guttmejer, W Michalski, M Bebenek, M Kryj. *Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer.* Br J Surg. **2006** Oct;93(10):1215-23. doi: 10.1002/bjs.5506.

10. Tadas Latkauskas, Henrikas Pauzas, Laura Kairevice, Aleksandras Petrauskas, Zilvinas Saladzinskas, Rasa Janciauskiene, Jurate Gudaityte, Paulius Lizdenis, Saulius Svagzdys, Algimantas Tamelis, Dainius Pavalkis. *Preoperative conventional chemoradiotherapy versus short-course radiotherapy with delayed surgery for rectal cancer: results of a randomized controlled trial.* BMC Cancer. **2016** Dec 1;16(1):927. doi: 10.1186/s12885-016-2959-9.

11. Emmanouil Fokas, Torsten Liersch, Rainer Fietkau, Werner Hohenberger, Tim Beissbarth, Clemens Hess, Heinz Becker, Michael Ghadimi, Karl Mrak, Susanne Merkel, Hans-Rudolf Raab, Rolf Sauer, Christian Wittekind, Claus Rödel. *Tumour regression grading after preoperative chemoradiotherapy for locally advanced rectal carcinoma revisited: updated results of the CAO/ARO/AIO-94 trial.* J Clin Oncol. **2014** May 20;32(15):1554-62. doi: 10.1200/JCO.2013.54.3769.

12. Emmanouil Fokas, Philipp Ströbel, Rainer Fietkau, Michael Ghadimi, Torsten Liersch, Gerhard G Grabenbauer, Arndt Hartmann, Marco Kaufmann, Rolf Sauer, Ullrich Graeven, Hans Hoffmanns, Hans-Rudolf Raab, Torsten Hothorn, Christian Wittekind, Claus Rödel, German Rectal Cancer Study Group. *Tumour Regression Grading After Preoperative Chemoradiotherapy as a Prognostic Factor and Individual-Level Surrogate for Disease-Free Survival in Rectal Cancer.* J Natl Cancer Inst. **2017** Dec 1;109(12). doi: 10.1093/jnci/djx095.

13. E Salmo, Y El-Dhuwaib, N Y Haboubi. *Histological grading of tumour regression and radiation colitis in locally advanced rectal cancer following neoadjuvant therapy: a critical appraisal*. Colorectal Dis. **2011** Oct;13(10):1100-6. doi: 10.1111/j.1463-1318.2010.02412.x.

14. Nicolas Leupin, Jürgen Curschmann, Helmut Kranzbühler, Christoph A Maurer, Jean A Laissue, Luca Mazzucchelli. *Acute radiation colitis in patients treated with short-term preoperative radiotherapy for rectal cancer*. Am J Surg Pathol. **2002** Apr;26(4):498-504. doi: 10.1097/00000478-200204000-00013.

15. P A Hall, D P Lane. *p53 in tumour pathology: can we trust immunohistochemistry? Revisited!* J Pathol. **1994** Jan;172(1):1-4. doi: 10.1002/path.1711720103.

16. Anna Yemelyanova, Russell Vang, Malti Kshirsagar, Dan Lu, Morgan A Marks, Ie Ming Shih, Robert J Kurman. *Immunohistochemical staining patterns of p53 can serve as a surrogate marker for TP53 mutations in ovarian carcinoma: an immunohistochemical and nucleotide sequencing analysis*. Mod Pathol. **2011** Sep;24(9):1248-53. doi: 10.1038/modpathol.2011.85.

17. Anna Colomer, Nadina Erill, Montse Verdú, Ruth Roman, August Vidal, Carlos Cordon-Cardo, Xavier Puig. *Lack of p53 nuclear immunostaining is not indicative of absence of TP53 gene mutations in colorectal adenocarcinomas*. Appl Immunohistochem Mol Morphol. **2003** Jun;11(2):130-7. doi: 10.1097/00129039-200306000-00007.

18. International Agency for Research on Cancer (IARC). Global Cancer Observatory. Cancer Today. **2020**. https://gco.iarc.fr/today/online-analysis-pie?v=2020&mode=cancer&mode_population=continents&population=900&populations=900&key=total&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=7&group_cancer=1&include_nmsc=1&include_nmsc_other=1&half_pie=0&donut=0. Accessed 20 December 2020.

19. International Agency for Research on Cancer (IARC). Global Cancer Observatory. Cancer Today. **2020**. https://gco.iarc.fr/today/online-analysis-pie?v=2020&mode=cancer&mode_population=regions&population=900&populations=900&key=total&sex=1&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=7&group_cancer=1&include_nmsc=1&include_nmsc_other=1&half_pie=0&donut=0. Accessed 20 December 2020.

20. International Agency for Research on Cancer (IARC). Global Cancer Observatory. Cancer Today. **2020**. https://gco.iarc.fr/today/online-analysis-pie?v=2020&mode=cancer&mode_population=regions&population=900&populations=900&key=total&sex=2&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=7&group_cancer=1&include_nmsc=1&include_nmsc_other=1&half_pie=0&donut=0. Accessed 20 December 2020.

21. Camilla Mattiuzzi, Fabian Sanchis-Gomar, Giuseppe Lippi. *Concise update on colorectal cancer epidemiology*. Ann Transl Med. **2019** Nov; 7(21): 609. doi: 10.21037/atm.2019.07.91.

22. Prashanth Rawla, Tagore Sunkara, Adam Barsouk. *Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors*. Prz Gastroenterol. **2019**;14(2):89-103. doi: 10.5114/pg.2018.81072.

23. Caitlin C Murphy, Robert S Sandler, Hanna K Sanoff, Y Claire Yang, Jennifer L Lund, John A Baron. *Decrease in Incidence of Colorectal Cancer Among Individuals 50 Years or Older After Recommendations for Population-based Screening*. Clin Gastroenterol Hepatol. **2017** Jun;15(6):903-909.e6. doi: 10.1016/j.cgh.2016.08.037.

24. Rebecca L Siegel, Stacey A Fedewa, William F Anderson, Kimberly D Miller, Jiemin Ma, Philip S Rosenberg, Ahmedin Jemal. *Colorectal Cancer Incidence Patterns in the United States, 1974-2013*. J Natl Cancer Inst. **2017** Aug 1;109(8):djw322. doi: 10.1093/jnci/djw322.

25. Christina E Bailey, Chung-Yuan Hu, Y Nancy You, Brian K Bednarski, Miguel A Rodriguez-Bigas, John M Skibber, Scott B Cantor, George J Chang. *Increasing disparities in the age-related incidences of colon and rectal cancers in the United States, 1975-2010*. JAMA Surg. **2015** Jan;150(1):17-22. doi: 10.1001/jamasurg.2014.1756.

26. Elena M Stoffel, Caitlin C Murphy. *Epidemiology and Mechanisms of the Increasing Incidence of Colon and Rectal Cancers in Young Adults*. Gastroenterology. **2020** Jan;158(2):341-353. doi: 10.1053/j.gastro.2019.07.055.

27. Caitlin C Murphy, Amit G Singal, John A Baron, Robert S Sandler. *Decrease in Incidence of Young-Onset Colorectal Cancer Before Recent Increase*. Gastroenterology. **2018** Dec;155(6):1716-1719.e4. doi: 10.1053/j.gastro.2018.07.045.

28. Darren R Brenner, Yibing Ruan, Eileen Shaw, Prithwish De, Steven J Heitman, Robert J Hilsden. *Increasing colorectal cancer incidence trends among younger adults in Canada*. Prev Med. **2017** Dec;105:345-349. doi: 10.1016/j.ypmed.2017.10.007.

29. Eleonora Feletto, Xue Qin Yu, Jie-Bin Lew, D James B St John, Mark A Jenkins, Finlay A Macrae, Suzanne E Mahady, Karen Canfell. *Trends in Colon and Rectal Cancer Incidence in Australia from 1982 to 2014: Analysis of Data on Over 375,000 Cases*. Cancer Epidemiol Biomarkers Prev. **2019** Jan;28(1):83-90. doi: 10.1158/1055-9965.EPI-18-0523.

30. Roger Yat-Nork Chung, Kelvin K F Tsoi, Moe Htet Kyaw, Abdul Rashid Lui, Francisco T T Lai, Joseph Jao-Yiu Sung. *A population-based age-period-cohort study of colorectal cancer incidence comparing Asia against the West*. Cancer Epidemiol. **2019** Apr;59:29-36. doi: 10.1016/j.canep.2019.01.007.

31. Yang Y, Land KC. *Age-Period-Cohort Analysis: New Models, Methods, and Empirical Applications*. Boca Raton, FL: CRC Press, **2013**.

32. Caitlin C Murphy, Kristin Wallace, Robert S Sandler, John A Baron. *Racial Disparities in Incidence of Young-Onset Colorectal Cancer and Patient Survival*. Gastroenterology. **2019** Mar;156(4):958-965. doi: 10.1053/j.gastro.2018.11.060.

33. Khalid Saad El Din, Jonathan M Loree, Eric C Sayre, Sharlene Gill, Carl J Brown, Hallie Dau, Mary A De Vera. *Trends in the epidemiology of young-onset colorectal cancer: a worldwide systematic review*. BMC Cancer. **2020** Apr 6;20(1):288. doi: 10.1186/s12885-020-06766-9.

34. Jessica B O'Connell, Melinda A Maggard, Edward H Livingston, Cifford K Yo. *Colorectal cancer in the young*. Am J Surg. **2004** Mar;187(3):343-8. doi: 10.1016/j.amjsurg.2003.12.020.

35. International Agency for Research on Cancer (IARC). Global Cancer Observatory. Cancer Today. **2020**. https://gco.iarc.fr/tomorrow/en/dataviz/bars?group_cancers=1&multiple_cancers=1&cancers=8_9&single_unit=100000&years=2040&types=1&mode=population&sort_by=value0&populations=903_904_905_908_909_935_900. Accessed 20 December 2020.

36. International Agency for Research on Cancer (IARC). Global Cancer Observatory. Cancer Today. **2020**. https://gco.iarc.fr/tomorrow/en/dataviz/bars?group_cancers=1&multiple_cancers=1&cancers=8_9&single_unit=100000&years=2040&types=1&mode=population&sort_by=value0&populations=984_983_982. Accessed 20 December 2020.

37. Melina Arnold, Mónica S Sierra, Mathieu Laversanne, Isabelle Soerjomataram, Ahmedin Jemal, Freddie Bray. *Global patterns and trends in colorectal cancer incidence and mortality*. Gut. **2017** Apr;66(4):683-691. doi: 10.1136/gutjnl-2015-310912.

38. Brenda K Edwards, Elizabeth Ward, Betsy A Kohler, Christie Eheman, Ann G Zauber, Robert N Anderson, Ahmedin Jemal, Maria J Schymura, Iris Lansdorp-Vogelaar, Laura C Seeff, Marjolein van Ballegooijen, S Luuk Goede, Lynn A G Ries *Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates*. Cancer. **2010** Feb 1;116(3):544-73. doi: 10.1002/cncr.24760.

39. Siân A Pugh, Bethany Shinkins, Alice Fuller, Jane Mellor, David Mant, John N Primrose. *Site and Stage of Colorectal Cancer Influence the Likelihood and Distribution*

of Disease Recurrence and Postrecurrence Survival: Data From the FACS Randomized Controlled Trial. Ann Surg. **2016** Jun;263(6):1143-7. doi: 10.1097/SLA.0000000000001351.

40. B Cady, M D Stone, J Wayne. *Continuing trends in the prevalence of right-sided lesions among colorectal carcinomas*. Arch Surg. **1993** May;128(5):505-9. doi: 10.1001/archsurg.1993.01420170035004.

41. A G M T Powell, R Wallace, R F McKee, J H Anderson, J J Going, J Edwards, P G Horgan. *The relationship between tumour site, clinicopathological characteristics and cancer-specific survival in patients undergoing surgery for colorectal cancer*. Colorectal Dis. **2012** Dec;14(12):1493-9. doi: 10.1111/j.1463-1318.2012.03048.x.

42. A J Greenstein, G Slater, T M Heimann, D B Sachar, and A H Aufses, Jr. *A comparison of multiple synchronous colorectal cancer in ulcerative colitis, familial polyposis coli, and de novo cancer*. Ann Surg. **1986** Feb; 203(2): 123–128. doi: 10.1097/00000658-198602000-00002.

43. H Iishi, M Tatsuta, S Tsutsui, K Imanishi, T Otani, S Okuda, S Ishiguro, H Taniguchi. *Early depressed adenocarcinomas of the large intestine*. Cancer. **1992** May 15;69(10):2406-10. doi: 10.1002/1097-0142(19920515)69:10<2406::aid-cncr2820691004>3.0.co;2-e.

44. Aejaz Nasir, David Boulware, Hans E Kaiser, Bela Bodey, Stuart Siegel, Stephanie Crawley, Timothy Yeatman, Jorge E Marcet, Domenico Coppola. *Flat and polypoid adenocarcinomas of the colorectum: A comparative histomorphologic analysis of 47 cases*. Hum Pathol. **2004** May;35(5):604-11. doi: 10.1016/j.humpath.2003.11.011.

45. Kazuhiro Yasuda, Yoichi Ajioka, Hidenobu Watanabe, Keiji Matsuda, Seigo Kitand. *Morphogenesis and development of superficial spreading tumour of the colon and rectum*. Pathol Int. **1997**;47(11):769-774. doi: 10.1111/j.1440-1827.1997.tb04455.x.

46. Mee Joo, Aliakbar Shahsafaei, Robert D Odze. *Paneth cell differentiation in colonic epithelial neoplasms: evidence for the role of the Apc/beta-catenin/Tcf pathway*. Hum Pathol. **2009** Jun;40(6):872-80. doi: 10.1016/j.humpath.2008.12.003.

47. L De Smedt, J Lemahieu, S Palmans, O Govaere, T Tousseyn, E Van Cutsem, H Prenen, S Tejpar, M Spaepen, G Matthijs, C Decaestecker, X Moles Lopez, P Demetter, I Salmon, X Sagaert. *Microsatellite instable vs stable colon carcinomas: analysis of tumour heterogeneity, inflammation and angiogenesis*. Br J Cancer. **2015** Jul 28;113(3):500-9. doi: 10.1038/bjc.2015.213.

48. Annalisa Legitimo, Rita Consolini, Alessandra Failli, Giulia Orsini, Roberto Spisni. *Dendritic cell defects in the colorectal cancer*. Hum Vaccin Immunother. **2014**;10(11):3224-35. doi: 10.4161/hv.29857.

49. K Tajima, M Yamakawa, Y Inaba, T Katagiri, H Sasaki. *Cellular localization of interleukin-5 expression in rectal carcinoma with eosinophilia*. Hum Pathol. **1998** Sep;29(9):1024-8. doi: 10.1016/s0046-8177(98)90212-x.

50. M Abdulkader, K Abdulla, E Rakha, P Kaye. *Routine elastic staining assists detection of vascular invasion in colorectal cancer*. Histopathology. **2006** Nov;49(5):487-92. doi: 10.1111/j.1365-2559.2006.02533.x.

51. H Zhang, A Ahmadi, G Arbman, J Zdolsek, J Carstensen, B Nordenskjöld, P Söderkvist, X F Sun. *Glutathione S-transferase T1 and M1 genotypes in normal mucosa, transitional mucosa and colorectal adenocarcinoma*. Int J Cancer. **1999** Apr 20;84(2):135-8. doi: 10.1002/(sici)1097-0215(19990420)84:2<135::aid-ijc7>3.0.co;2-c.

52. Marc R Lewin, Hubert Fenton, Ashlie L Burkart, Todd Sheridan, Amer K Abu-Alfa, Elizabeth A Montgomery. *Poorly differentiated colorectal carcinoma with invasion restricted to lamina propria (intramucosal carcinoma): a follow-up study of 15 cases*. Am J Surg Pathol. **2007** Dec;31(12):1882-6. doi: 10.1097/PAS.0b013e318057fac2.

53. Y Cao, P M Schlag, U Karsten. *Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colonic epithelium: apolar localization corresponds to malignant transformation*. Virchows Arch. **1997** Sep;431(3):159-66. doi: 10.1007/s004280050083.

54. Michael D Walsh, Joanne P Young, Barbara A Leggett, Stephanie H Williams, Jeremy R Jass, Michael A McGuckin. *The MUC13 cell surface mucin is highly expressed by human colorectal carcinomas*. Hum Pathol. **2007** Jun;38(6):883-92. doi: 10.1016/j.humpath.2006.11.020.

55. Michael D Walsh, Mark Clendenning, Elizabeth Williamson, Sally-Ann Pearson, Rhiannon J Walters, Belinda Nagler, David Packenas, Aung K Win, John L Hopper, Mark A Jenkins, Andrew M Haydon, Christophe Rosty, Dallas R English, Graham G Giles, Michael A McGuckin, Joanne P Young, Daniel D Buchanan. *Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype*. Mod Pathol. **2013** Dec;26(12):1642-56. doi: 10.1038/modpathol.2013.101.

56. K Berezowski, J F Stastny, M J Kornstein. *Cytokeratins 7 and 20 and carcinoembryonic antigen in ovarian and colonic carcinoma*. Mod Pathol. **1996** Apr;9(4):426-9.

57. T S Loy, R D Calaluce, G L Keeney. *Cytokeratin immunostaining in differentiating primary ovarian carcinoma from metastatic colonic adenocarcinoma*. Mod Pathol. **1996** Nov;9(11):1040-4.

58. A I Kende, N J Carr, L H Sobin. *Expression of cytokeratins 7 and 20 in carcinomas of the gastrointestinal tract*. Histopathology. **2003** Feb;42(2):137-40. doi: 10.1046/j.1365-2559.2003.01545.x.

59. Reda S Saad, Jan F Silverman, Mahmoud A Khalifa, Corwyn Rowsell. *CDX2, cytokeratins 7 and 20 immunoreactivity in rectal adenocarcinoma*. Appl Immunohistochem Mol Morphol. **2009** May;17(3):196-201. doi: 10.1097/PAI.0b013e31819268f2.

60. M J O'Brien, N Zamcheck, B Burke, S E Kirkham, C A Saravis, L S Gottlieb. *Immunocytochemical localization of carcinoembryonic antigen in benign and malignant colorectal tissues. Assessment of diagnostic value*. Am J Clin Pathol. **1981** Mar;75(3):283-90. doi: 10.1093/ajcp/75.3.283.

61. Y Hamada, M Yamamura, K Hioki, M Yamamoto, H Nagura, K Watanabe. *Immunohistochemical study of carcinoembryonic antigen in patients with colorectal cancer. Correlation with plasma carcinoembryonic antigen levels*. Cancer. **1985** Jan 1;55(1):136-41. doi: 10.1002/1097-0142(19850101)55:1<136::aid-cncr2820550121>3.0.co;2-p.

62. Vassil Kaimaktchiev, Luigi Terracciano, Luigi Tornillo, Hanspeter Spichtin, Dimitra Stoios, Marcel Bundi, Veselina Korcheva, Martina Mirlacher, Massimo Loda, Guido Sauter, Christopher L Corless. *The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas*. Mod Pathol. **2004** Nov;17(11):1392-9. doi: 10.1038/modpathol.3800205.

63. Robert W Werling, Hadi Yaziji, Carlos E Bacchi, Allen M Gown. *CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas*. Am J Surg Pathol. **2003** Mar;27(3):303-10. doi: 10.1097/00000478-200303000-00003.

64. M B Listrom, J V Little, M McKinley, C M Fenoglio-Preiser. *Immunoreactivity of tumour-associated glycoprotein (TAG-72) in normal, hyperplastic, and neoplastic colon*. Hum Pathol. **1989** Oct;20(10):994-1000. doi: 10.1016/0046-8177(89)90271-2.

65. C E Bacchi, A M Gown. *Distribution and pattern of expression of villin, a gastrointestinal-associated cytoskeletal protein, in human carcinomas: a study employing paraffin-embedded tissue*. Lab Invest. **1991** Mar;64(3):418-24.

66. E Campo, J Muñoz, R Miquel, A Palacín, A Cardesa, B F Sloane, M R Emmert-Buck. *Cathepsin B expression in colorectal carcinomas correlates with tumour progression and shortened patient survival*. Am J Pathol. **1994** Aug;145(2):301-9.

67. Donna E Hansel, Robert E Wilentz, Charles J Yeo, Richard D Schulick, Elizabeth Montgomery, Anirban Maitra. *Expression of neuropilin-1 in high-grade dysplasia, invasive cancer, and metastases of the human gastrointestinal tract*. Am J Surg Pathol. **2004** Mar;28(3):347-56. doi: 10.1097/00000478-200403000-00007.

68. Min-Cheng Su, Ray-Hwang Yuan, Chiao-Ying Lin, Yung-Ming Jeng. *Cadherin-17 is a useful diagnostic marker for adenocarcinomas of the digestive system*. Mod Pathol. **2008** Nov;21(11):1379-86. doi: 10.1038/modpathol.2008.107.

69. V Gotzos, E S Wintergerst, J P Musy, H P Spichtin, C Y Genton. *Selective distribution of calretinin in adenocarcinomas of the human colon and adjacent tissues*. Am J Surg Pathol. **1999** Jun;23(6):701-11. doi: 10.1097/00000478-199906000-00010.

70. A Yamaguchi, T Ishida, G Nishimura, T Kumaki, M Katoh, T Kosaka, Y Yonemura, I Miyazaki. *Human chorionic gonadotropin in colorectal cancer and its relationship to prognosis*. Br J Cancer. **1989** Sep;60(3):382-4. doi: 10.1038/bjc.1989.289.

71. E Campo, A Palacin, C Benasco, E Quesada, A Cardesa. *Human chorionic gonadotropin in colorectal carcinoma. An immunohistochemical study*. Cancer. **1987** May 1;59(9):1611-6. doi: 10.1002/1097-0142(19870501)59:9<1611::aid-cncr2820590915>3.0.co;2-h.

72. M L Slattery, W S Samowitz, J A Holden. *Estrogen and progesterone receptors in colon tumours*. Am J Clin Pathol. **2000** Mar;113(3):364-8. doi: 10.1309/5MHB-K6XX-QV50-PCJQ.

73. Zong-Ming E Chen, Jon H Ritter, Hanlin L Wang. *Differential expression of alpha-methylacyl coenzyme A racemase in adenocarcinomas of the small and large intestines*. Am J Surg Pathol. **2005** Jul;29(7):890-6. doi: 10.1097/01.pas.0000167364.90899.59.

74. Sandra Meršaková, Zora Lasabová, Ján Strnádel, Michal Kalman, Eva Gabonova, Peter Sabaka, Rachele Ciccocioppo, Luis Rodrigo, Peter Kruzliak, Peter Mikolajčík. *Genomic profile and immune contexture in colorectal cancer-relevance for prognosis and immunotherapy*. Clin Exp Med. **2020** Jul 27. doi: 10.1007/s10238-020-00649-w.

75. E R Fearon, B Vogelstein. *A genetic model for colorectal tumourigenesis*. Cell. **1990** Jun 1;61(5):759-67. doi: 10.1016/0092-8674(90)90186-i.

76. Cancer Genome Atlas Network. *Comprehensive molecular characterization of human colon and rectal cancer*. Nature. **2012** Jul 18;487(7407):330-7. doi: 10.1038/nature11252.

77. Hans Raskov, Jacob H Søby, Jesper Troelsen, Rasmus D Bojesen, Ismail Gögenur. *Driver Gene Mutations and Epigenetics in Colorectal Cancer*. Ann Surg. **2020** Jan;271(1):75-85. doi: 10.1097/SLA.0000000000003393.

78. Bert Vogelstein, Kenneth W Kinzler. *The Path to Cancer --Three Strikes and You're Out*. N Engl J Med. **2015** Nov 12;373(20):1895-8. doi: 10.1056/NEJMp1508811.

79. Cristian Tomasetti, Luigi Marchionni, Martin A Nowak, Giovanni Parmigiani, Bert Vogelstein. *Only three driver gene mutations are required for the development of lung and colorectal cancers*. Proc Natl Acad Sci U S A. **2015** Jan 6;112(1):118-23. doi: 10.1073/pnas.1421839112.

80. Michael J Schell, Mingli Yang, Jamie K Teer, Fang Yin Lo, Anup Madan, Domenico Coppola, Alvaro N A Monteiro, Michael V Nebozhyn, Binglin Yue, Andrey Loboda, Gabriel A Bien-Willner, Danielle M Greenawalt, Timothy J Yeatman. *A multigene mutation classification of 468 colorectal cancers reveals a prognostic role for APC*. Nat Commun. **2016** Jun 15;7:11743. doi: 10.1038/ncomms11743.

81. Joseph R, Little P, Hayes DN, et al. *Characterization of the number and site of APC mutations in sporadic colorectal cancer*. J Clin Oncol. **2017**;35:630–1630.

82. Kevin M Haigis. *KRAS Alleles: The Devil Is in the Detail*. Trends Cancer. **2017** Oct;3(10):686-697. doi: 10.1016/j.trecan.2017.08.006.

83. Dongdong Huang, Wenjie Sun, Yuwei Zhou, Peiwei Li, Fang Chen, Hanwen Chen, Dajing Xia, Enping Xu, Maode Lai, Yihua Wu, Honghe Zhang. *Mutations of key driver genes in colorectal cancer progression and metastasis*. Cancer Metastasis Rev. **2018** Mar;37(1):173-187. doi: 10.1007/s10555-017-9726-5.

84. Karin M Hardiman. *Update on Sporadic Colorectal Cancer Genetics*. Clin Colon Rectal Surg. **2018** May;31(3):147-152. doi: 10.1055/s-0037-1602234.

85. Hae Su Kim, Jin Seok Heo, Jeeyun Lee, Ji Yun Lee, Min-Young Lee, Sung Hee Lim, Woo Yong Lee, Seok Hyung Kim, Yoon Ah Park, Yong Beom Cho, Seong Hyeon Yun, Seung Tae Kim, Joon Oh Park, Ho Yeong Lim, Yong Soo Choi, Woo Il Kwon, Hee Cheol Kim, Young Suk Park. *The impact of KRAS mutations on prognosis in surgically*

resected colorectal cancer patients with liver and lung metastases: a retrospective analysis. BMC Cancer. **2016** Feb 18;16:120. doi: 10.1186/s12885-016-2141-4.

86. David Barras, Sevtap Savas. *Biomarkers for colon cancer*. Biomark Cancer. **2015**; 7(Suppl 1): 49–50. doi: 10.4137/BIC.S39882

87. Thomas JS, Shi C. *Molecular Testing in Colorectal Cancer*. Cambridge, USA: Academic Press Inc; **2016**.

88. E Sanz-Garcia, G Argiles, E Elez, J Tabernero. *BRAF mutant colorectal cancer: prognosis, treatment, and new perspectives*. Ann Oncol. **2017** Nov 1;28(11):2648-2657. doi: 10.1093/annonc/mdx401.

89. Yujun Hao, Yardena Samuels, Qingling Li, Dawid Krokowski, Bo-Jhih Guan, Chao Wang, Zhicheng Jin, Bohan Dong, Bo Cao, Xiujing Feng, Min Xiang, Claire Xu, Stephen Fink, Neal J Meropol, Yan Xu, Ronald A Conlon, Sanford Markowitz, Kenneth W Kinzler, Victor E Velculescu, Henri Brunengraber, Joseph E Willis, Thomas LaFramboise, Maria Hatzoglou, Guo-Fang Zhang, Bert Vogelstein, Zhenghe Wang. *Oncogenic PIK3CA mutations reprogram glutamine metabolism in colorectal cancer*. Nat Commun. **2016** Jun 20;7:11971. doi: 10.1038/ncomms11971.

90. Aoife J McCarthy, Runjan Chetty. *Smad4/DPC4*. J Clin Pathol. **2018**;71:661-664. doi:10.1136/jclinpath-2018-205095

91. Isaac Wasserman, Lik Hang Lee, Shuji Ogino, Michael R Marco, Chao Wu, Xi Chen, Jashodeep Datta, Eran Sadot, Bryan Szeglin, Jose G Guillem, Philip B Paty, Martin R Weiser, Garrett M Nash, Leonard Saltz, Afsar Barlas, Katia Manova-Todorova, Srijaya Prakash Babu Uppada, Arthur E Elghouayel, Peter Ntiamoah, Jonathan N Glickman, Tsuyoshi Hamada, Keisuke Kosumi, Kentaro Inamura, Andrew T Chan, Reiko Nishihara, Andrea Cercek, Karuna Ganesh, Nancy E Kemeny, Punita Dhawan, Rona Yaeger, Charles L Sawyers, Julio Garcia-Aguilar, Marios Giannakis, Jinru Shia, J Joshua Smith. *SMAD4 Loss in Colorectal Cancer Patients Correlates with Recurrence, Loss of Immune Infiltrate, and Chemoresistance*. Clin Cancer Res. **2019** Mar 15;25(6):1948-1956. doi: 10.1158/1078-0432.CCR-18-1726. Epub 2018 Dec 26.

92. Tao Jiang, Ling Ye, Zhongbo Han, Yuan Liu, Yinxue Yang, Zhihai Peng, Junwei Fan. *miR-19b-3p promotes colon cancer proliferation and oxaliplatin-based chemoresistance by targeting SMAD4*: validation by bioinformatics and experimental analyses. J Exp Clin Cancer Res. **2017** Sep 22;36(1):131. doi: 10.1186/s13046-017-0602-5.

93. Cyriac Kandoth, Michael D McLellan, Fabio Vandin, Kai Ye, Beifang Niu, Charles Lu, Mingchao Xie, Qunyuan Zhang, Joshua F McMichael, Matthew A Wyczalkowski, Mark D M Leiserson, Christopher A Miller, John S Welch, Matthew J Walter, Michael C Wendl, Timothy J Ley, Richard K Wilson, Benjamin J Raphael, Li Ding. *Mutational landscape and significance across 12 major cancer types*. Nature. **2013** Oct 17;502(7471):333-339. doi: 10.1038/nature12634.

94. Lijun Du, John J Kim, Jinhua Shen, Binrui Chen, Ning Dai. *KRAS and TP53 mutations in inflammatory bowel disease-associated colorectal cancer: a meta-analysis*. Oncotarget. **2017** Mar 28;8(13):22175-22186. doi: 10.18632/oncotarget.14549.

95. Hans Raskov, Hans-Christian Pommergaard, Jakob Burcharth, Jacob Rosenberg. *Colorectal carcinogenesis--update and perspectives*. World J Gastroenterol. **2014** Dec 28;20(48):18151-64. doi: 10.3748/wjg.v20.i48.18151.

96. John M Carethers. *Differentiating Lynch-like from Lynch syndrome*. Gastroenterology. **2014** Mar;146(3):602-4. doi: 10.1053/j.gastro.2014.01.041. Epub 2014 Jan 24.

97. J Olsen, M L M Espersen, P Jess, L T Kirkeby, J T Troelsen. *The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review*. Surg Oncol. **2014** Sep;23(3):167-76. doi: 10.1016/j.suronc.2014.07.003. Epub 2014 Jul 17.

98. Janina Graule, Kristin Uth, Elia Fischer, Irene Centeno, José A Galván, Micha Eichmann, Tilman T Rau, Rupert Langer, Heather Dawson, Ulrich Nitsche, Peter Traeger, Martin D Berger, Beat Schnüriger, Marion Hädrich, Peter Studer, Daniel Inderbitzin, Alessandro Lugli, Mario P Tschan, Inti Zlobec. *CDX2 in colorectal cancer is an independent prognostic factor and regulated by promoter methylation and histone deacetylation in tumours of the serrated pathway*. Clin Epigenetics. **2018** Sep 26;10(1):120. doi: 10.1186/s13148-018-0548-2.

99. Yasuyuki Shigematsu, Kentaro Inamura, Yoshihiro Mise, Akio Saiura, Emil Rehnberg, Noriko Yamamoto, Yuichi Ishikawa, Shunji Takahashi, Hiroaki Kanda. *CDX2 expression is concordant between primary colorectal cancer lesions and corresponding liver metastases independent of chemotherapy: a single-center retrospective study in Japan*. Oncotarget. **2018** Mar 30;9(24):17056-17065. doi: 10.18632/oncotarget.24842.

100. Yunshuai Wang, Zhaohui Li, Wenxian Li, Shuaifeng Liu, Baowei Han. *Methylation of promoter region of CDX2 gene in colorectal cancer*. Oncol Lett. **2016** Nov;12(5):3229-3233. doi: 10.3892/ol.2016.5109. Epub 2016 Sep 9.

101. Piero Dalerba, Debashis Sahoo, Soonmyung Paik, Xiangqian Guo, Greg Yothers, Nan Song, Nate Wilcox-Fogel, Erna Forgó, Pradeep S Rajendran, Stephen P Miranda, Shigeo Hisamori, Jacqueline Hutchison, Tomer Kalisky, Dalong Qian, Norman Wolmark, George A Fisher, Matt van de Rijn, Michael F Clarke. *CDX2 as a Prognostic Biomarker in Stage II and Stage III Colon Cancer*. N Engl J Med. **2016** Jan 21;374(3):211-22. doi: 10.1056/NEJMoa1506597.

102. Keyan Salari, Mary E Spulak, Justin Cuff, Andrew D Forster, Craig P Giacomini, Stephanie Huang, Melissa E Ko, Albert Y Lin, Matt van de Rijn, Jonathan R Pollack. *CDX2 is an amplified lineage-survival oncogene in colorectal cancer*. Proc Natl Acad Sci U S A. **2012** Nov 13;109(46):E3196-205. doi: 10.1073/pnas.1206004109. Epub 2012 Oct 29.

103. Yitzhak Reizel, Ofra Sabag, Yael Skversky, Adam Spiro, Benjamin Steinberg, Diana Bernstein, Amber Wang, Julia Kieckhaefer, Catherine Li, Eli Pikarsky, Rena Levin-Klein, Alon Goren, Klaus Rajewsky, Klaus H Kaestner, Howard Cedar. *Postnatal DNA demethylation and its role in tissue maturation*. Nat Commun. **2018** May 23;9(1):2040. doi: 10.1038/s41467-018-04456-6.

104. Yuval Dor, Howard Cedar. *Principles of DNA methylation and their implications for biology and medicine*. Lancet. **2018** Sep 1;392(10149):777-786. doi: 10.1016/S0140-6736(18)31268-6. Epub 2018 Aug 9.

105. Elisa Danese, Martina Montagnana. *Epigenetics of colorectal cancer: emerging circulating diagnostic and prognostic biomarkers*. Ann Transl Med. **2017** Jul;5(13):279. doi: 10.21037/atm.2017.04.45.

106. H Lin, Y Zhang, H Wang, D Xu, X Meng, Y Shao, C Lin, Y Ye, H Qian, S Wang. *Tissue inhibitor of metalloproteinases-3 transfer suppresses malignant behaviors of colorectal cancer cells*. Cancer Gene Ther. **2012** Dec;19(12):845-51. doi: 10.1038/cgt.2012.70. Epub 2012 Oct 5.

107. Claire Gallois, Simon Pernot, Aziz Zaanan, Julien Taieb. *Colorectal Cancer: Why Does Side Matter?* Drugs. **2018** Jun;78(8):789-798. doi: 10.1007/s40265-018-0921-7.

108. Molly A Wasserman, Michael F McGee, Irene B Helenowski, Amy L Halverson, Anne-Marie Boller, Steven J Stryker. *The anthropometric definition of the rectum is*

highly variable. Int J Colorectal Dis. **2016** Feb;31(2):189-95. doi: 10.1007/s00384-015-2458-5. Epub 2015 Nov 25.

109. Y Nancy You, Karin M Hardiman, Andrea Bafford, Vitaliy Poylin, Todd D Francone, Kurt Davis, Ian M Paquette, Scott R Steele, Daniel L Feingold, Clinical Practice Guidelines Committee of the American Society of Colon and Rectal Surgeons. *The American Society of Colon and Rectal Surgeons Clinical Practice Guidelines for the Management of Rectal Cancer*. Dis Colon Rectum. **2020** Sep;63(9):1191-1222. doi: 10.1097/DCR.0000000000001762.

110. Nigel D'Souza, Michael P M de Neree Tot Babberich, Andre d'Hoore, Emmanuel Tiret, Evaghelos Xynos, Regina G H Beets-Tan, Iris D Nagtegaal, Lennart Blomqvist, Torbjorn Holm, Bengt Glimelius, Antonio Lacy, Andres Cervantes, Robert Glynne-Jones, Nicholas P West, Rodrigo O Perez, Claudio Quadros, Kil Yeon Lee, Thandinkosi E Madiba, Steven D Wexner, Julio Garcia-Aguilar, Dushyant Sahani, Brendan Moran, Paris Tekkis, Harm J Rutten, Pieter J Tanis, Theo Wiggers, Gina Brown. *Definition of the Rectum: An International, Expert-based Delphi Consensus*. Ann Surg. **2019** Dec;270(6):955-959. doi: 10.1097/SLA.0000000000003251.

111. R Glynne-Jones, L Wyrwicz, E Tiret, G Brown, C Rödel, A Cervantes, D Arnold, ESMO Guidelines Committee. *Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up*. Ann Oncol. **2017** Jul 1;28(suppl_4):iv22-iv40. doi: 10.1093/annonc/mdx224.

112. Joakim Folkesson, Helgi Birgisson, Lars Pahlman, Bjorn Cedermark, Bengt Glimelius, Ulf Gunnarsson. *Swedish Rectal Cancer Trial: long lasting benefits from radiotherapy on survival and local recurrence rate*. J Clin Oncol. **2005** Aug 20;23(24):5644-50. doi: 10.1200/JCO.2005.08.144.

113. Mark S Roh, Linda H Colangelo, Michael J O'Connell, Greg Yothers, Melvin Deutsch, Carmen J Allegra, Morton S Kahlenberg, Luis Baez-Diaz, Carol S Ursiny, Nicholas J Petrelli, Norman Wolmark. *Preoperative multimodality therapy improves disease-free survival in patients with carcinoma of the rectum: NSABP R-03*. J Clin Oncol. **2009** Nov 1;27(31):5124-30. doi: 10.1200/JCO.2009.22.0467. Epub 2009 Sep 21.

114. Rolf Sauer, Torsten Liersch, Susanne Merkel, Rainer Fietkau, Werner Hohenberger, Clemens Hess, Heinz Becker, Hans-Rudolf Raab, Marie-Therese Villanueva, Helmut Witzigmann, Christian Wittekind, Tim Beissbarth, Claus Rödel. *Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the*

German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. J Clin Oncol. **2012** Jun 1;30(16):1926-33. doi: 10.1200/JCO.2011.40.1836. Epub 2012 Apr 23.

115. David Sebag-Montefiore, Richard J Stephens, Robert Steele, John Monson, Robert Grieve, Subhash Khanna, Phil Quirke, Jean Couture, Catherine de Metz, Arthur Sun Myint, Eric Bessell, Gareth Griffiths, Lindsay C Thompson, Mahesh Parmar. *Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial*. Lancet. **2009** Mar 7;373(9666):811-20. doi: 10.1016/S0140-6736(09)60484-0.

116. Willem van Gijn, Corrie A M Marijnen, Iris D Nagtegaal, Elma Meershoek-Klein Kranenbarg, Hein Putter, Theo Wiggers, Harm J T Rutten, Lars Påhlman, Bengt Glimelius, Cornelis J H van de Velde, Dutch Colorectal Cancer Group. *Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial*. Lancet Oncol. **2011** Jun;12(6):575-82. doi: 10.1016/S1470-2045(11)70097-3. Epub 2011 May 17.

117. Iosief Abraha, Cynthia Aristei, Isabella Palumbo, Marco Lupattelli, Stefano Trastulli, Roberto Cirocchi, Rita De Florio, Vincenzo Valentini. *Preoperative radiotherapy and curative surgery for the management of localised rectal carcinoma*. Cochrane Database Syst Rev. **2018** Oct 3;10(10):CD002102. doi: 10.1002/14651858.CD002102.pub3.

118. C Cammà, M Giunta, F Fiorica, L Pagliaro, A Craxì, M Cottone. *Preoperative radiotherapy for resectable rectal cancer: A meta-analysis*. JAMA. **2000** Aug 23-30;284(8):1008-15. doi: 10.1001/jama.284.8.1008.

119. Nuh N Rahbari, Heike Elbers, Vasileios Askoxylakis, Edith Motschall, Ulrich Bork, Markus W Büchler, Jürgen Weitz, Moritz Koch. *Neoadjuvant radiotherapy for rectal cancer: meta-analysis of randomized controlled trials*. Ann Surg Oncol. **2013** Dec;20(13):4169-82. doi: 10.1245/s10434-013-3198-9. Epub 2013 Sep 4.

120. Koen C M J Peeters, Corrie A M Marijnen, Iris D Nagtegaal, Elma Klein Kranenbarg, Hein Putter, Theo Wiggers, Harm Rutten, Lars Pahlman, Bengt Glimelius, Jan Willem Leer, Cornelis J H van de Velde, Dutch Colorectal Cancer Group. *The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma*. Ann Surg. **2007** Nov;246(5):693-701. doi: 10.1097/01.sla.0000257358.56863.ce.

121. Jennifer Y Wo, Christopher J Anker, Jonathan B Ashman, Nishin A Bhadkamkar, Lisa Bradfield, Daniel T Chang, Jennifer Dorth, Julio Garcia-Aguilar, David Goff, Dustin Jacqmin, Patrick Kelly, Neil B Newman, Jeffrey Olsen, Ann C Raldow, Erika Ruiz-Garcia, Karyn B Stitzenberg, Charles R Thomas Jr, Q Jackie Wu, Prajnan Das. *Radiation Therapy for Rectal Cancer: Executive Summary of an ASTRO Clinical Practice Guideline*. Pract Radiat Oncol. **2020** Oct 21;S1879-8500(20)30207-1. doi: 10.1016/j.prro.2020.08.004. Online ahead of print.

122. Martin Loos, Philipp Quentmeier, Tibor Schuster, Ulrich Nitsche, Ralf Gertler, Andreas Keerl, Thomas Kocher, Helmut Friess, Robert Rosenberg. *Effect of preoperative radio(chemo)therapy on long-term functional outcome in rectal cancer patients: a systematic review and meta-analysis*. Ann Surg Oncol. **2013** Jun;20(6):1816-28. doi: 10.1245/s10434-012-2827-z. Epub 2012 Dec 27.

123. Bin Ma, Peng Gao, Hongchi Wang, Qingzhou Xu, Yongxi Song, Xuanzhang Huang, Jingxu Sun, Junhua Zhao, Junlong Luo, Yu Sun, Zhenning Wang. *What has preoperative radio(chemo)therapy brought to localized rectal cancer patients in terms of perioperative and long-term outcomes over the past decades? A systematic review and meta-analysis based on 41,121 patients*. Int J Cancer. **2017** Sep 1;141(5):1052-1065. doi: 10.1002/ijc.30805. Epub 2017 Jun 8.

124. Helgi Birgisson, Lars Påhlman, Ulf Gunnarsson, Bengt Glimelius, Swedish Rectal Cancer Trial Group. *Adverse effects of preoperative radiation therapy for rectal cancer: long-term follow-up of the Swedish Rectal Cancer Trial*. J Clin Oncol. **2005** Dec 1;23(34):8697-705. doi: 10.1200/JCO.2005.02.9017.

125. E Kapiteijn, C A Marijnen, I D Nagtegaal, H Putter, W H Steup, T Wiggers, H J Rutten, L Pahlman, B Glimelius, J H van Krieken, J W Leer, C J van de Velde, Dutch Colorectal Cancer Group. *Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer*. N Engl J Med. **2001** Aug 30;345(9):638-46. doi: 10.1056/NEJMoa010580.

126. K C M J Peeters, C J H van de Velde, J W H Leer, H Martijn, J M C Junggeburt, E Klein Kranenbarg, W H Steup, T Wiggers, H J Rutten, C A M Marijnen. *Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients--a Dutch colorectal cancer group study*. J Clin Oncol. **2005** Sep 1;23(25):6199-206. doi: 10.1200/JCO.2005.14.779.

127. Lisette M Wiltink, Tina Y T Chen, Remi A Nout, Elma Meershoek-Klein Kranenbarg, Marta Fiocco, Søren Laurberg, Cornelis J H van de Velde, Corrie A M Marijnen. *Health-related quality of life 14 years after preoperative short-term radiotherapy and total mesorectal excision for rectal cancer: report of a multicenter randomised trial*. Eur J Cancer. **2014** Sep;50(14):2390-8. doi: 10.1016/j.ejca.2014.06.020. Epub 2014 Jul 21.

128. Nabila Ansari, Michael J Solomon, Richard J Fisher, John Mackay, Bryan Burmeister, Stephen Ackland, Alexander Heriot, David Joseph, Sue-Anne McLachlan, Bev McClure, Samuel Y Ngan. *Acute Adverse Events and Postoperative Complications in a Randomized Trial of Preoperative Short-course Radiotherapy Versus Long-course Chemoradiotherapy for T3 Adenocarcinoma of the Rectum: Trans-Tasman Radiation Oncology Group Trial (TROG 01.04)*. Ann Surg. **2017** May;265(5):882-888. doi: 10.1097/SLA.0000000000001987.

129. K Bujko, M P Nowacki, L Kepka, J Oledzki, M Bebenek, M Kryj, Polish Colorectal Study Group. *Postoperative complications in patients irradiated pre-operatively for rectal cancer: report of a randomised trial comparing short-term radiotherapy vs chemoradiation*. Colorectal Dis. **2005** Jul;7(4):410-6. doi: 10.1111/j.1463-1318.2005.00796.x.

130. Zhi-Rui Zhou, Shi-Xin Liu, Tian-Song Zhang, Ling-Xiao Chen, Jun Xia, Zhi-De Hu, Bo Li. *Short-course preoperative radiotherapy with immediate surgery versus long-course chemoradiation with delayed surgery in the treatment of rectal cancer: a systematic review and meta-analysis*. Surg Oncol. **2014** Dec;23(4):211-21. doi: 10.1016/j.suronc.2014.10.003. Epub 2014 Oct 29.

131. Yongjun Yu, Yuwei Li, Chen Xu, Zhao Zhang, Xipeng Zhang. *Comparison of long course and short course preoperative radiotherapy in the treatment of locally advanced rectal cancer: a systematic review and meta-analysis*. Rev Esp Enferm Dig. **2019** Jan;111(1):17-27. doi: 10.17235/reed.2018.5674/2018.

132. Johan Erlandsson, Torbjörn Holm, David Pettersson, Åke Berglund, Björn Cedermark, Calin Radu, Hemming Johansson, Mikael Machado, Fredrik Hjern, Olof Hallböök, Ingvar Syk, Bengt Glimelius, Anna Martling. *Optimal fractionation of preoperative radiotherapy and timing to surgery for rectal cancer (Stockholm III): a multicentre, randomised, non-blinded, phase 3, non-inferiority trial*. Lancet Oncol. **2017** Mar;18(3):336-346. doi: 10.1016/S1470-2045(17)30086-4. Epub 2017 Feb 10.

133. D Pettersson, E Lörinc, T Holm, H Iversen, B Cedermark, B Glimelius, A Martling. *Tumour regression in the randomized Stockholm III Trial of radiotherapy regimens for rectal cancer*. Br J Surg. **2015** Jul;102(8):972-8; discussion 978. doi: 10.1002/bjs.9811.

134. Laura De Caluwé, Yves Van Nieuwenhove, Wim P Ceelen. *Preoperative chemoradiation versus radiation alone for stage II and III resectable rectal cancer.* Cochrane Database Syst Rev. **2013** Feb 28;(2):CD006041. doi: 10.1002/14651858.CD006041.pub3.

135. Wang Qiaoli, Huang Yongping, Xiong Wei, Xu Guoqiang, Ju Yunhe, Liu Qiuyan, Li Cheng, Guo Mengling, Li Jiayi, Xiong Wei, Yang Yi. *Preoperative short-course radiotherapy (5 × 5 Gy) with delayed surgery versus preoperative long-course radiotherapy for locally resectable rectal cancer: a meta-analysis*. Int J Colorectal Dis. **2019** Dec;34(12):2171-2183. doi: 10.1007/s00384-019-03433-9. Epub 2019 Nov 19.

136. O Dworak, L Keilholz, A Hoffmann. *Pathological features of rectal cancer after preoperative radiochemotherapy*. Int J Colorectal Dis. **1997**;12(1):19-23. doi: 10.1007/s003840050072.

137. Montgomery, E.A.; Oshima, K.; Voltaggio, L. *Survival Guide to gastrointestinal mucosal biopsies. Pathology Survival Guide First Series-Vol. 1,* 1st ed.; Innovative Pathology Press: Arlington, VA, USA, **2017**.

138. T P Brien, F A Farraye, R D Odze. *Gastric dysplasia-like epithelial atypia associated with chemoradiotherapy for esophageal cancer: a clinicopathologic and immunohistochemical study of 15 cases*. Mod Pathol. **2001** May;14(5):389-96. doi: 10.1038/modpathol.3880323.

139. Fabio Nocera, Fiorenzo Angehrn, Markus von Flüe, Daniel C Steinemann. *Optimising functional outcomes in rectal cancer surgery*. Langenbecks Arch Surg. **2020** Jul 26. doi: 10.1007/s00423-020-01937-5. Online ahead of print.

140. Ha Thi Nguyen, Hong-Quan Duong. *The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy*. Oncol Lett. **2018** Jul;16(1):9-18. doi: 10.3892/ol.2018.8679. Epub 2018 May 9.

141. Colin J R Stewart, Simon Hillery. *Mucosal endocrine cell micronests and single endocrine cells following neo-adjuvant therapy for adenocarcinoma of the distal oesophagus and oesophagogastric junction*. J Clin Pathol. **2007** Nov;60(11):1284-9. doi: 10.1136/jcp.2007.047449. Epub 2007 Sep 24.

142. Jinru Shia, Satish K Tickoo, Jose G Guillem, Jing Qin, Aviram Nissan, Axel Hoos, Alexander Stojadinovic, Leyo Ruo, W Douglas Wong, Philip B Paty, Martin R Weiser, Bruce D Minsky, David S Klimstra. *Increased endocrine cells in treated rectal adenocarcinomas: a possible reflection of endocrine differentiation in tumour cells induced by chemotherapy and radiotherapy*. Am J Surg Pathol. **2002** Jul;26(7):863-72. doi: 10.1097/00000478-200207000-00004.

Acknowledgements

I thank the Pathology Unit (Dr. Alberto Cavazza), Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy, for the financial support.
I thank Dr. Magda Zanelli and Dr. Loredana De Marco (Pathology Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy) for the histopathological and immunohistochemical analysis as well as for the histopathological images.
I thank Dr. Alessia Ciarrocchi and the Laboratory of Translational Research, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy, for the NGS analysis.
I thank Dr. Davide Nicoli and the Laboratory of Molecular Biology, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy, for the biomolecular analysis.
I thank Dr. Dafne Ramundo and the Radiotherapy Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy, for the description of the preoperative radiotherapy protocol for LARC.
I thank Dr. Massimo Costantini (Scientific Director, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy) for the constant support received during these years of PhD, and without which it would not have been possible to start, develop and increase the quantity and quality of scientific research.
I thank Prof. Giuseppe Biagini, (PhD Programme Coordinator, Università di Modena e Reggio Emilia, Italy) for the appreciated professionalism with which he managed to develop a PhD course of great interest and characterized by engaging and passionate teachers.
I thank Prof. Filippo Lococo (Thoracic Surgery, Fondazione Policlinico Universitario A. Gemelli IRCCS, Italy) for his invaluable professional support and his sincere friendship.
I thank Dr. Daniela Masi (Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy) for English editing.

I thank my parents, my brother and my sister for their ever constant love.