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Molecular Heterogeneity and Prognostic Implications of Synchronous Advanced Colorectal Neoplasia

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ABSTRACT

Background and Aim. *It is uncertain whether synchronous colorectal cancers (S-CRC) develop through a preferential carcinogenetic pathway and whether they have a poorer prognosis. Since microsatellite unstable tumors (MSI) might act as a bias when assessing methylation and outcome of S-CRC, we analysed for synchronous neoplasia and for disease-specific survival (DSS) a large series of CRC systematically characterized by BRAF and MS status.*

Patients and Methods. *881 patients consecutively resected for CRC and perioperatively investigated with complete colonoscopy, were included in the study. For each patient, demographic and clinico-pathological records were retrieved and DSS was retrospectively assessed. All cancers were screened for MSI by BAT 26/BAT25 mononucleotide analysis. MSI tumors had the MMR protein defect identified at immunohistochemistry (IHC) and were tested for germline mutation of the corresponding gene. All tumors were also analysed for BRAF $^{c.1799T>A}$ mutation.*

Results. *S-CRC was associated with stage IV (p=0.01) and with HNPCC (p<0.001), but only MSS S-CRC accounted for the association with metastatic CRC (p=0.001). BRAF mutation was associated with sporadic MSI CRC (p<0.001) but not with S-CRC (p=0.96). S-CRC did not affect DSS in patients with MSI CRC (HR 0.74; 95%CI 0.09-5.75; p=0.77). Conversely, S-CRC (HR 1.82; 95%CI 1.15-2.87;p=0.01), as well as synchronous advanced adenoma (HR 1.81; 95%CI 1.27-2.58; p=0.001), and BRAF $^{c.1799T>A}$ mutation (HR 2.16; 95%CI 1.25-3.73; p=0.01) were stage-independent predictors of death related to MSS CRC.*

Conclusions. *Microsatellite-stable CRC have a worse prognosis if S-CRC, or even a synchronous advanced adenoma, is diagnosed. Neither the occurrence of multiple MSS advanced neoplasia nor their enhanced aggressiveness are mediated by an epigenetic field effect. Surveillance and therapeutic protocols for MSS CRC should take into account the prognostic burden of synchronous advanced colorectal neoplasms.*

SOMMARIO

***Introduzione e scopo**. In letteratura molte ipotesi sono state formulate sull'insorgenza dei tumori sincroni del colon retto (S-CRC). Infatti, non è ancora del tutto chiaro se questo tipo di malattia sviluppa attraverso un particolare pathway molecolare legato ad una elevata suscettibilità della mucosa colica a divenire neoplastica e se a livello clinico evidenzia una prognosi peggiore. Dal momento che i tumori con instabilità dei microsatelliti (MSI) possono costituire un bias nel valutare la presenza di metilazione e l'andamento clinico dei S-CRC, abbiamo analizzato per neoplasia sincrona e per disease-specific survival (DSS), una ampia casistica di CRC caratterizzata per la mutazione di BRAF e per lo stato dei microsatelliti. **Pazienti e Metodi**. Nello studio sono stati arruolati 881 pazienti consecutivi, i quali sono stati sottoposti a resezione del cancro del colon e hanno eseguito a livello perioperatorio una colonscopia completa. Per ogni paziente, i dati demografici e clinico-patologici sono stati recuperati e il DSS è stato valutato in modo retrospettivo. Tutti i tumori sono stati sottoposti a screening per MSI, per identificare pazienti con HNPCC, mediante l'analisi di marcatori mononucleotidici come BAT 26 e BAT25. Nei tumori con instabilità dei microsatelliti, il difetto proteico è stato individuato mediante immunoistochimica (IHC) e successivamente sono stati testati per la mutazione germinale del gene corrispondente. Inoltre, tutti i tumori sono stati anche analizzati per la mutazione di BRAF $^{c.1799T>A}$. **Risultati.** Il S-CRC è stato associato con lo stadio IV (p = 0.01) e con l'HNPCC (p <0.001), ma solo i casi MSS S-CRC sono più frequentemente metastatici (p = 0.001). La mutazione di BRAF $^{c.1799T>A}$ era associata con gli MSI CRC sporadici (p <0.001), ma non con i S-CRC (p = 0.96). I cancri sincroni non cambiano il miglior DSS dei pazienti con MSI CRC (HR 0.74, 95% CI 0.09-5.75, p = 0.77). Viceversa, i MSS S-CRC (HR 1.82, 95% CI 1.15-2.87, p = 0.01), così come un tumore MSS e la presenza di un adenoma avanzato (sincrono adenoma avanzato) (HR 1.81, 95% CI 1.27-2.58, p = 0.001), e la mutazione di BRAF $^{c.1799T>A}$ (HR 2.16, 95% CI 1.25-3.73, p = 0.01) risultano essere variabili predittori di un aumentato rischio di morte indipendenti dallo stadio di malattia. **Conclusioni.** I MSS S-CRC evidenziano una prognosi peggiore, e lo stesso vale per la presenza di un sincrono adenoma avanzato. Dallo studio emerge che né la presenza di multiple avanzate neoplasie in tumori MSS né la loro maggiore aggressività sono mediati da un effetto epigenetico. Per cui la sorveglianza e i protocolli terapeutici per i pazienti con un MSS CRC dovrebbero tener conto del peso prognostico dato dalla presenza di neoplasie sincrone avanzate del colon-retto.*

INDEX

ABBREVIATIONS

APC= Adenomatous Polyposis Coli

CIMP= CpG Island Methylator Phenotype

CIN= Cromosomal Instability

CRC= Colorectal Cancer

FAP= Familial Adenomatous Polyposis

HNPCC= Hereditary Non Polyposis Colorectal Cancer

HP= Hyperplastic polyp

LOH= Loss of Heterozygosity

MAP= MYH-Associated Polyposis

MSI= Microsatellite Instability

MSS= Microsatellite Stability

TSA= Traditional Serrated Adenoma(

S-CRC= Synchronous Colorectal Cancer

SSA= Sessile Serrated Adenoma

1. INTRODUCTION

1.1 Colorectal Cancer

1.1.1 Epidemiology

Colorectal cancer (CRC) is the second leading cause of cancer death in Western countries [2-4], with a mortality rate of 30 cases per 100,000 inhabitants in Europe each year [5]. More than 90% of CRC new cases are diagnosed in people over the age of 50 [6]. In men, CRC is the most common tumor second only to adenocarcinoma prostate with an average age of onset of about 68 years [1]. Meanwhile in women is the third most frequent tumor after breast and uterus cancers [1]. with an average onset age of about 72 years [1].

More than one million new cases are diagnosed worldwide each year [7], and in Europe the incidence is about 58 cases per 100,000 people [5]. Over the past twenty years a decline in CRC incidence and mortality was observed [3, 6]. In particular from 1990 to 2005 the death rate was significantly reduced (-31.8% for men and -28% for women) [3], from 50% in 2000 [6] to 33% in 2010 [7]. The reduction in the incidence rate may depend not only from the intensification of screening methods use [6], but also from increased aspirin and other FANS assumption, and postmenopausal hormone replacement therapy introduction in women, both protective factors for the occurrence of CRC [3].

The frequency of the CRC is very variable depending on the cohort analyzed. Indeed it have been demonstrated that geographical CRC incidence differences exist [9]. A possible explanations include the distribution of some regional or local characteristics of patients (race, ethnicity, culture), modifiable lifestyle (smoking, diet, exercise) and theoretically the genetic risk factors of CRC [8, 9]. In particular, African American population shows a higher incidence and mortality [8]. Moreover, in literature it has been suggested that the socio-economic status could influence CRC risk. Therefore, the measure of poverty status of a geographical area may explain the regional variations in the incidence and survival of cancer [8]. Regarding the location of the tumor, in the 80s it has been observed a reduction in the left colon (descending, sigmoid colon, rectum) CRC incidence, which are also more frequent in the under 70 years, but not in CRC in the right side (ascending colon, caecum).

This was attributed to the use of endoscopic screening by recto-sigmoidoscopy (RSS) and colonoscopy (PCS). Indeed, the full PCS is strongly associated with a decrease in distal tumors mortality (OR 0.33, but not in proximal tumors death (OR 0.99), probably due to biological differences between the proximal and distal colon and to a higher number of lesions not identified in the right colon side [2].

1.1.2 Prognostic Factors

In many types of tumors the extent or stage of cancer at the time of diagnosis is a key factor that defines prognosis and it is a critical element in determining appropriate treatment [11]. The most clinically useful and important staging system is the Tumor Node Metastasis (TNM) system.

This system is the result of close collaboration between the International Union Against Cancer (UICC-TNM) and the American Joint Committee on Cancer (AJCC). TNM system was initially developed only to predict prognosis, but its application was expanded also to establish the suitable treatment and to select the patients to include in for clinical trials [12].

The TNM system classifies cancers by the primary tumor size and extention (T), the involvement of regional lymph node (N), and the presence or absence of distant metastases (M). However, the criteria for defining anatomic extent of disease are specific for the tumors with different anatomic sites and different histological types [11].

The TNM staging classification include:

- clinical TNM (cTNM) is defined by information obtained from symptoms; physical examination; endoscopic examinations; imaging studies of the tumor, regional lymph nodes, and metastases; biopsies of the primary tumor; and surgical exploration without resection.
- pathological TNM (pTNM) is defined by the same diagnostic studies used for clinical staging supplemented by findings from surgical resection and histologic examination of the surgically removed tissues.
- post therapy or post neoadjuvant therapy TNM (yTNM) includes systemic or radiation treatment prior to surgical resection.

In CRC, the TNM staging remains the gold standard of prognostic factor and play an important role in determining the 5-years survival rate in these patients [5]. Despite many editions of TNM have been proposed by the years, those after the fifth edition did not provide significant and adequate advantage in CRC staging [13].

Staging of CRC is based on the depth of tumor penetration through the bowel wall or adherence to adjacent organs or structures (T), degree of presence of regional lymph node involvement (N) and presence or absence of distant metastasis (M) (**Figure 1**).

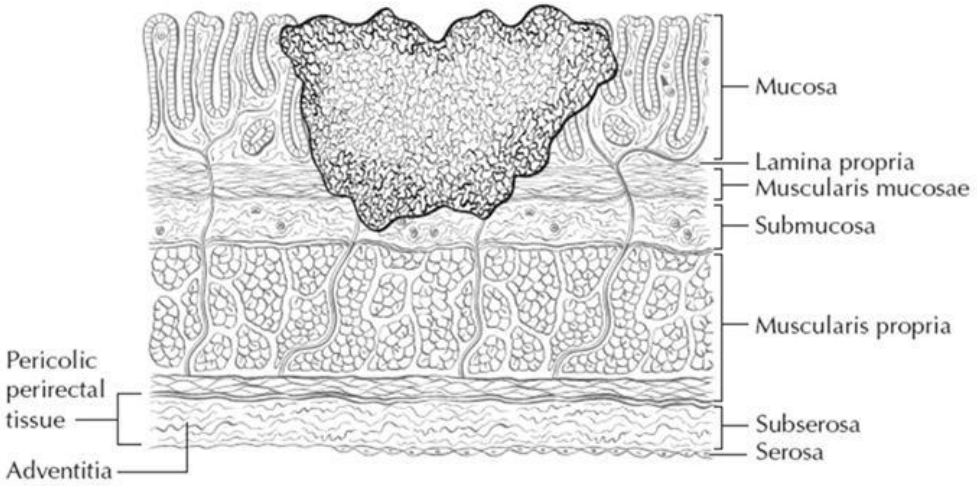

Figure 1 The different components of colic mucosa involved during tumor invasion.

According to the fifth edition of the TNM staging system, the depth of tumor invasion is classified by:

pT is refers to the presence of malignant cells confined above the basement membrane or lamina propria;

pT1 tumor invades submucosa;

pT2 tumor invades muscularis propria;

pT3 tumor invades through the muscularis propria into the subserosa or into nonperitonealized pericolic tissues;

pT4 tumor directly invades other organs or structures and/or perforates visceral peritoneum;

The lymph node involvement is classified by:

N0 no regional lymph node metastasis;

N1 metastasis to one to three regional lymph nodes;

N2 metastasis to four or more regional lymph nodes;

The presence or absence of distant metastasis is classified by

M0 no distant metastasis;

M1 distant metastasis;

Patients with a similar prognosis, T, N and M are grouped into so-called anatomic stage/prognostic groups, commonly referred to as stage and this classification with respect to the fifth edition of the American Joint Committee on Cancer (AJCC) system [14] in the (**Table I**).

Stage	Local Invasion	Nodal Invasion	Distant Metastasis
I	T1	N0	M0
	T2	N0	M0
II	T3	N0	M0
	T4	N0	M0
III	Any T	N1	M0
IV	Any T	Any N	M1

***Table I CRC stages classified according to the fifth edition of the American Joint Committee on Cancer (AJCC) system*.**

In addition to TNM stage, other tumor-related features such as venous and lymphatic invasion, tumor grade, and tumor budding have been identified as essential or important prognostic factors [5].

Venous and lymphatic invasion represents crucial steps in the formation of micro-metastases and eventually macroscopic tumor growth at a secondary site.

Tumor grade is based on the percentage of gland formation.

- well-differentiated (G1) adenoma with more than 95% of glands within the tumor;
- moderately differentiated (G2) with 50 to 95% of glandular structure;
- poorly differentiated (G3) with 5 to 50% of glandular structure;
- undifferentiated (G4) carcinoma with less than 5% of glandular structure [15].

Tumor budding is described as a transition from glandular structures to single cells or clusters of up to four cells at the invasive margin of CRC with malignant potential. It has been show that the presence of “buds” at the tumor invasive front represents an independent predictor of lymph node metastasis in patients with sub-mucosal invasive or early pT1 CRCs and probably the frequency of tumor budding increases with more advanced TNM stage [16]. Therefore, molecular and immunohistochemical analysis have been proposed to improve the prognostic value, although at present the tumor stage is still the most important prognostic factor in CRC.

1.1.3 Polyps

Polyps are most common in the colon but may occur in the esophagus, stomach, and small intestine.

Intestinal polyps can be classified as non-neoplastic or neoplastic in nature. The common neoplastic polyp is adenoma while the non-neoplastic polyps can be further classified as inflammatory, hamartomatous, or hyperplastic [17].

Many colorectal cancer develops from adenomas, the precursor lesions, which are benign intraepithelial neoplasms composed of dysplastic cells with malignant potential. The risk of cancer developing within an adenoma increases with size, grade of dysplasia and villosity. These features, together with polyp numbers, are also predictive of metachronous neoplasia and may therefore influence the decision to offer follow-up endoscopic surveillance [17].

The presence of one or more colorectal adenomas in the different populations depends on age, gender and family history. In fact, the prevalence rates in asymptomatic individuals ≥ 50 years, determined by colonoscopy, range from 24% to 50% while the presence of advanced adenomas (≥ 1 cm in size with villous features, and/or with high grade dysplasia) varies from 3.4 up to 9.5% [18].

The incidence of adenomas at intervals ranging from 6 months to 5 years in post-polypectomy surveillance colonoscopy studies varies from 20 to 50%. The most incident polyps are small, and a higher incidence has been associated with multiple adenomas at the index colonoscopy, larger size of the index adenoma, older age, and a family history of a parent with colorectal cancer. A large studies have demonstrated that regular surveillance colonoscopy and polyps removal reduce the incidence of colorectal adenocarcinoma [18].

Adenomas are characterized according to macroscopic and histological features. Gross features: pedunculated; sessile and flat [15].

- Pedunculated adenomas appear as exophytic, mucosal protrusions with a lobulated head and a stalk covered by normal mucosa. The adenomatous epithelium remains confined to the mucosa of the head of the polyp. The stalk consists of normal mucosa, including the muscularis mucosae and submucosal tissue, in continuity with the major part of the bowel wall.
- Sessile adenomas attach to the mucosa by a broad base. They are often less well circumscribed than pedunculated ones. Because of their ill-defined edges, they are difficult to delineate, and have a greater tendency to recur following local excision.
- Flat adenomas are lesions that lack an exophytic polypoid configuration. They consist of slightly elevated dysplastic mucosal plaques that are never

- greater than twice the thickness of the surrounding normal colonic mucosa. They constitute a special subgroup of adenomas with a greater potential for malignant transformation, while still being smaller than exophytic adenomas.

Histological features: tubular; villous; tubulo-villous and grade of dysplasia [15].

- Tubular adenomas maintain the original crypt architecture, but adenomatous epithelium replaces the normal colonic epithelium in lining the crypts. This is the most common type of adenoma (about 68% to 87%). Tubular lesions are those that contain greater than the 80% of tubular component. Tubular adenomas consist of closely packed branching tubules separated by varying amounts of lamina propria. The tubule may be relatively regular, or when the adenomatous tubules grow, they may branch and show considerable irregularity.
- Villous adenomas (approximately 20%) have villi with cores of lamina propria covered by a single layer of adenomatous epithelium. Villous lesions are those that contain greater than 80% of a villous component. Villous adenomas fall into three types: flat, carpet-like masses; lobulated, bulky, sessile masses and pedunculated lesions with short, broad pedicles.
- Tubulo-villous adenomas contain a mixture of both tubular and villous patterns, or have broad villi containing short tubular structures. Tubulo-villous lesions are those that contain from 20% to 79% villous components. They tend to be larger than tubular adenomas, with a mean diameter of 19 mm and, usually, a villous component is present in 35% to 75% of all adenomas measuring more than 1 cm in largest diameter.

Grade of dysplasia: low-grade-dysplasia (LGD) and high-grade dysplasia (HGD) [18].

- Low grade dysplasia is characterized by tubules which are lined from the top to the bottom by epithelium which is morphologically similar to the normal basal proliferative zone. The nuclei are enlarged, oval, hyperchromatic, and have normal orientation. There is a slight excess of mitotic figures but the architecture is not disrupted.
- High grade dysplasia is characterized by large vesicular nuclei, irregular and conspicuous nucleoli, scalloped nuclear membranes, and increased nuclear to cytoplasmic ratio. Nuclear polarity is disrupted and marked cellular pleomorphism and both numerous and aberrant mitoses are present. Structural alterations include budding and branching tubules, back-to-back arrangement of glands, and cribriform growth of epithelial cells in clusters and sheets. This dysplasia encompasses the histological changes called carcinoma *in situ* and intramucosal carcinoma.

In addition, another group of polyps are represented by serrated polyps. In the past, all serrated polyps were classified simply as hyperplastic polyps and were considered to have no malignant potential. This group of polyps is comprised not only of hyperplastic polyps, but also of sessile serrated adenomas, traditional serrated adenomas and mixed polyps, showing serrated and classical adenomatous features [19].

- Hyperplastic polyp (HP) are by far the most common serrated polyps (80–90%). They occur most often in the distal part of the colon and rectum. Grossly, these are slightly elevated lesions with a diameter of usually less than 5 mm. Microscopically, hyperplastic polyps are characterized by elongated crypts with serrated architecture in the upper half of the crypts and sometimes, these changes can be detected only in the upper third and on the surface of the crypts. These polyps do not show cytological atypia or intraepithelial neoplasia. and structural or architectural changes but can be present genetic alterations.
- The traditional serrated adenoma (TSA) is the rarest variant of serrated lesions (1–6%). Grossly, TSAs are pedunculated or villous polyps, which are more common in the left side (60%) than in the right side of the colon in mostly elderly patients. Microscopically, TSA is characterized by 90% of low-grade-dysplasia and 10% of high-grade-dysplasia.
- The sessile serrated adenoma (SSA) is the second most common form of serrated polyps. Grossly, SSAs are flat or slightly elevated lesions typically >5 mm in diameter and localized in the right part of the colon. The microscopic characteristic of the SSA is hyperserration and dilatation of the crypts (with reduced stroma and back-to-back positioning of the dilated crypts) with T- and L-shaped branching at the crypt base.
- Mixed polyps are combinations of conventional (tubular, tubule-villous, and villous) adenomas with different grades of dysplasia and serrated lesions. There are different forms of mixed polyps according to their components: SSA and TSA, SSA and conventional adenoma, TSA and conventional adenoma, and rare combination as HP and conventional adenoma [19].

1.1.4 Molecular basis

The colorectal cancer develop through a “adenoma-carcinoma sequence” is characterized by a stepwise accumulation of genetic and epigenetic alterations, leading to the transformation of a normal colonic mucosa to an adenomatous intermediate and then ultimately adenocarcinoma [20].

Identification of different molecular pathways such as the chromosomal instability (CIN), the microsatellite instability (MSI), and the CpG Island Methylator Phenotype (CIMP) have demonstrated the heterogeneous nature of CRC.

1.1.4.1 Chromosomal instability (CIN or MSS)

CIN has been the first model of colorectal carcinogenesis proposed by Fearon and Vogelstein in 1990. In this multistep genetic model different genes are involved during onset, growth and development of CRC. Recent genome-wide sequencing efforts have calculated that more than 80 genes are mutated in colorectal tumors, but only a smaller group of mutations (<15) have been considered to be the true “drivers” of tumorigenesis.

CIN is observed in 80%-85% of sporadic colorectal cancers; and this type of the genomic instability shows an accelerated rate of gains or losses of whole or large portions of chromosomes that results in karyotypic variability from cell to cell. The consequence of CIN is an allelic imbalance at several chromosomal loci (including 5q, 8p, 17p, and 18q), subchromosomal genomic amplifications, and a high frequency of loss of heterozygosity (LOH) [20].

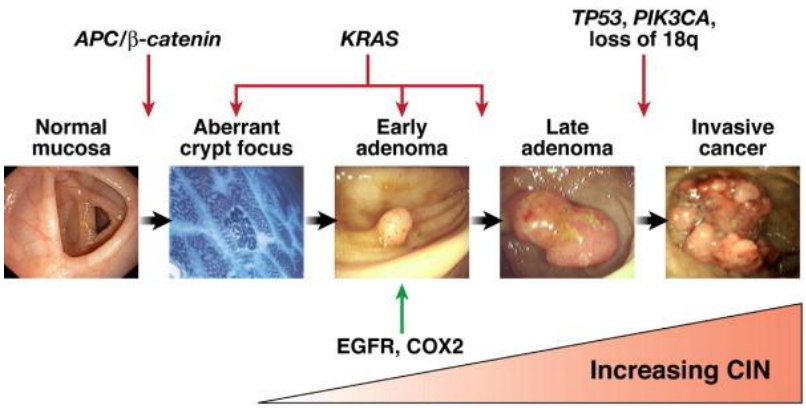

Figure 2 Multistep genetic model of colorectal carcinogenesis. (The Chromosomal instability in Colorectal cancer, Gastroenterology, 2010)

The first step is the inactivation of the *adenomatous polyposis coli* (APC) gene. APC mutations are observed in 5% of dysplastic aberrant crypt foci, 30%-70% of sporadic adenomas, and in as many as 72% of sporadic tumors, indicating that functional loss of APC is an early event in tumor initiation (**Figure 2**).

The APC gene product is a large protein with multiple functional domains that regulates differentiation, adhesion, polarity, migration, development, apoptosis, and even chromosomal segregation. Generally, APC binds to β-catenin, glycogen synthase kinase-3β, and casein kinase 1α/ε on an axin-conductin scaffold. The subsequent phosphorylation of β-catenin by glycogen synthase kinase-3β leads to proteasome-dependent degradation and suppression of the Wnt signal. The inactivation of APC leds to disruption of complex formation and to the increased cytoplasmic levels of β-catenin that can translocate to the nucleus, where it drives the transcription of multiple genes implicated in tumor growth and invasion through its interaction with the T-cell factor/lymphoid enhancer factor family of transcription factors [20].

The growth of adenoma is characterized by mutations in KRAS gene. KRAS is mutated in 30%-50% of CRCs and in 60%-95% of non-dysplastic or hyperplastic aberrant crypt foci, indicating that these microscopic lesions are unlikely precursors of adenomas and cancer [21-22]. The major single nucleotide point mutations occur in codons 12 and 13, and to a lesser extent in codon 61. Mutations in each of these three codons compromise the ability of GTPase-activating proteins leading to constitutive activation of RAS downstream signaling.

Activated RAS regulates multiple cellular functions through well-described effectors. The best characterized effector is the Raf–mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway. The Raf family includes 3 serine/ threonine kinases (ARAF, BRAF, and RAF1) that activate MEK1 and MEK2, which in turn phosphorylate ERK1 and ERK2. ERK then phosphorylates cytosolic and nuclear substrates, including JUN and ELK1, which regulate enzymes such as cyclin D1, which is involved in the control of cell cycle progression [23].

The growth progression and the adenomato-carcinoma transition is characterized by genetic alterations (mainly deletions) to genes on chromosome 18q, biallelic loss or inactivation of TP53 and in small proportion to mutational activation of PIK3CA.

Allelic loss at chromosome 18q has been identified in more than 70% of primary colorectal tumors, particularly in advanced stages and involved mainly in tumor suppressor genes such as SMAD2 and SMAD4.

This genes are intracellular mediators of the transforming growth factor-β pathway that are involved in the regulation of cell growth, differentiation, and apoptosis [24-25].

TP53 tumor suppressor gene is located on the short arm of chromosome 17 and the loss of function has been reported in 4%-26% of adenomas, 50% of adenomas with invasive foci, and in 50%-75% of CRC [26].

This gene encodes a 393 amino acid transcription factor that plays a central role in the controls of the transcription of genes involved in DNA metabolism, apoptosis, cell cycle regulation, senescence, angiogenesis, immune response, cell differentiation, motility, and migration [27-28].

1.1.4.2 Microsatellite instability (MSI)

MSI is a hypermutable phenotype caused by the loss of DNA mismatch repair system (MMR) activity. It is detected in about 15% of all colorectal cancers, the 3% are associated with Lynch syndrome also known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC) and the other 12% are caused by hypermethylation of the promoter of the MLH1 gene, which occurs in sporadic tumors with the CpG island methylator phenotype [29].

Microsatellites are repetitive sequences of DNA distributed throughout the human genome that consist of mono-, di-, trinucleotide or higher-order nucleotide repeats such as $(A)_n$, $(CA)_n$. [30]. DNA polymerase are error-prone enzymes, especially, in these sequences and inappropriate base insertion or DNA replication slippage at these sites results in insertion or deletion loops consisting of multiples of the nucleotide repeat sequence. These are normally repaired, but in absence of efficient MMR function, these loops become “fixed” and the next round of replication, result in alleles of different size [31] (**Figure 3**).

Instability of microsatellites is a reflection of the inability of the MMR system to correct these errors.

The MMR system is highly conserved from bacteria to humans and are responsible for maintaining genetic stability by repairing base to base mismatches and insertion/deletion loops (IDLs) that arise during S phase of DNA replication. In eukaryotic cells, MMR system include MutS and MutL proteins complex.

MutS family is characterized by MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3). These heterodimers have different relative abilities to bind to DNA mismatches and have a broader ability to recognize and repair different types of DNA misincorporation. In fact, MutSα has a higher affinity for recognizing single base-pair mismatches, while, MutSβ recognized with increased ability the IDLs [29].

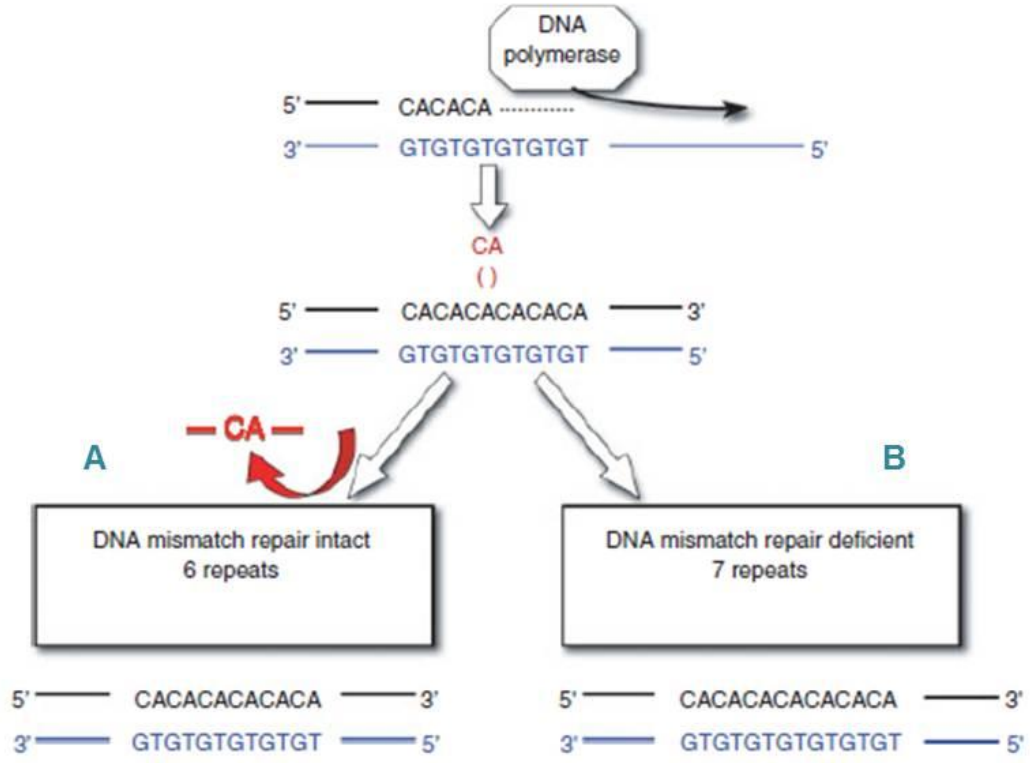

Figure 3 Mechanism of microsatellite instability. A) normal function of DNA mismatch repair the error is repaired; B) absence function of DNA mismatch repair the error is incorporated in the new strand of DNA. (Microsatellite instability in colorectal cancer, British journal of Surgery, 2006)

MutL family is characterized by MutLα (MLH1-PMS2), MutLβ (MLH1-PMS1), and MutLγ (MLH1-MLH3). MutLα mediates the interaction between the MutS proteins and enzymes involved in long-patch excision in post- replication mismatch repair, while, MutLβ suppresses mutagenesis in yeast but has an uncertain function in humans and MutLγ helps suppress IDL mutations and functions during meiosis in yeast, but its function in humans is unknown [29].

MMR process include other factors that help correct the errors and play a role in the initiation, DNA re-synthesis steps of the mismatch repair such as proliferating cell nuclear antigen (PCNA), single-strand DNA-binding protein (RPA), replication factor C (RF-C), exonuclease1 (EXO1), and DNA polymerase **(Figure 4**)

PCNA interacts with MSH2 and MLH1 and is thought to play roles in the initiation and DNA re-synthesis steps of MMR. PCNA also interacts with MSH6 and MSH3 via a conserved PCNA interaction motif termed the PIP box. It has been proposed that PCNA may help localize MutSα and MutSβ to mispairs in newly replicated DNA. Although PCNA is absolutely required during 3′ nick-directed MMR, it is not essential during 5′ nick-directed MMR. This observation might be explained by the fact that EXO1, a 5′→3′ exonuclease, is involved in both 5′ and 3′ directed MMR. Like PCNA, EXO1 also interacts with MSH2 and MLH1. While EXO1 can readily carry out 5′ directed mismatch excision in the presence of MutSα or MutSβ and single-strand DNA-binding protein (RPA), its role in catalyzing 3′ nick-directed excision requires the MutLα endonuclease, which is activated by PCNA and RFC. Although it has been suggested that EXO1 possesses a cryptic 3′→5′ exonuclease activity, current data do not support that hypothesis. Finally, the last steps consist in resynthesis of the excised strand, which requires the polymerases δ and DNA ligase I [32].

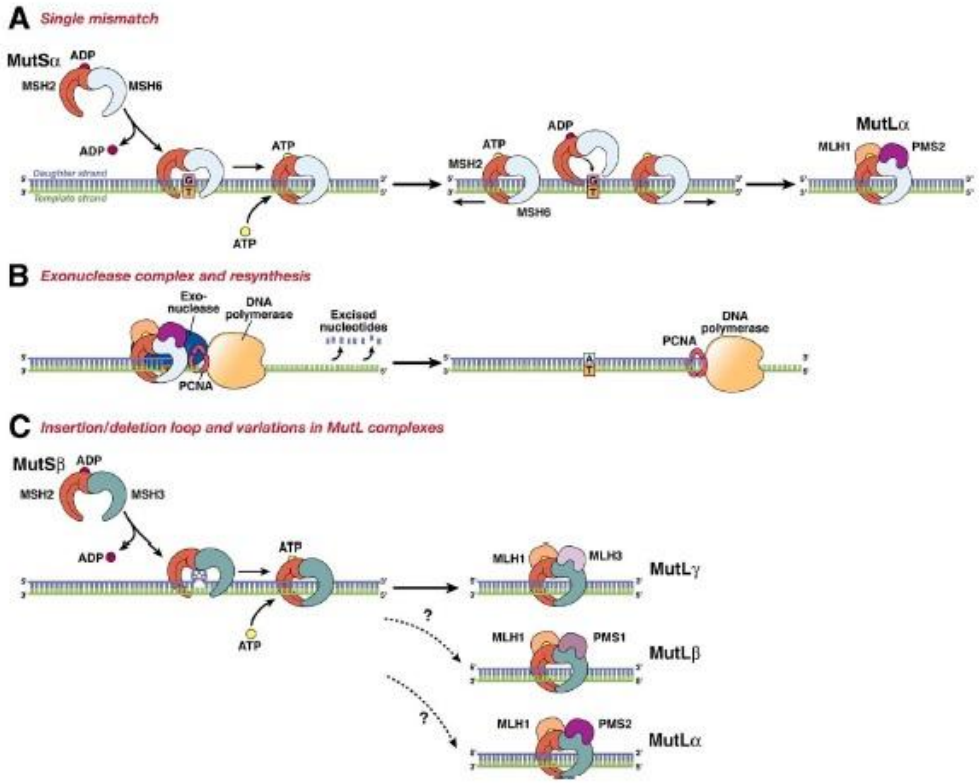

Figure 4 The DNA MMR system functions through a series of steps. A) MSH2–MSH6 (MutSα) recognizes single base-pair mismatches, in which the DNA polymerase has matched the wrong base (G) with the T on the template (shown on left), and creates a sliding clamp around the DNA. This step that requires the exchange of adenosine triphoshpate (ATP) for adenosine diphosphate (ADP) (by MSH2, but not MSH6 or MSH3). The complex diffuses away from the mismatch site, which is then bound by the MLH1-PMS2 (MutLα) complex (right). This "matchmaker" complex moves along the new DNA chain until it encounters the DNA polymerase complex. B) The DNA MMR protein sliding clamp interacts with exonuclease-1, proliferating cell nuclear antigen (PCNA), and DNA polymerase. This complex excises the daughter strand back to the site of the mismatch (shown on left). Eventually, the complex falls off the DNA and resynthesis occurs, correcting the error. C) Variations on the DNA MMR theme. Whereas MSH2–MSH6 recognizes single pair mismatches and small IDLs, MSH2–MSH3 (MutSβ) complements this by also recognizing larger IDLs (shown on left). The right side shows the possible interactions with different MutL dimers, as MLH1 can dimerize with PMS2, PMS1, or MLH3. The preferred interaction with MSH2–MSH3 is MLH1–MLH3 (MutLγ), but the precise roles of the other MutL heterodimers in this reaction are not entirely understood. (Microsatellite Instability in Colorectal cancer, Gastroenterology, 2010)

1.1.4.3 CpG Island Methylator Phenotype (CIMP)

Epigenetic alterations refer to changes in gene expression or function without changing the DNA sequence of that particular gene. In 1982, aberrant epigenetic alterations were first discovered in CRC. Since that time, research has revealed an 'epigenetic landscape' consisting of a complex array of epigenetic regulatory mechanisms that control gene expression in both normal tissue and cancerous tissue [33-34]. The epigenetic landscape is largely a reflection of factors that determine the condensation state of chromatin, which determines whether the DNA is accessible to proteins that control gene transcription. A relaxed or 'open' chromatin state allows for gene transcription, whereas a condensed or 'closed' chromatin state prevents gene transcription [35]. The epigenetic mechanisms currently believed to have a role in cancer development include: DNA methylation of cytosine bases in CG-rich sequences, called CpG islands; post-translational modification of histones (proteins that form the nucleosomes), which regulate the packaging structure of DNA (called chromatin); microRNAs and noncoding RNAs; and nucleosome positioning [35] (**Figure 5**).

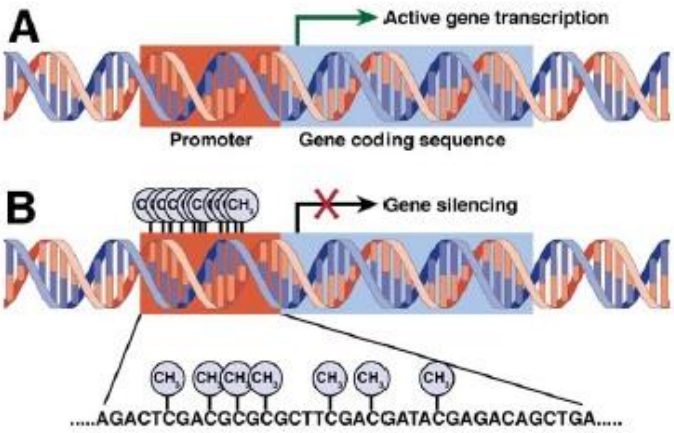

Figure 5 CpG island methylation leads to gene silencing. A)The gene promoter and coding regions of an active gene are indicated in red and blue, respectively. B) The aberrant addition of methyl groups (CH3) to CpG sites in the promoter region interferes with gene transcription, resulting in silencing. (Role of the Serrated Pathway in Colorectal Cancer Pathogenesis, Gastroenterology, 2010)

1.1.5 Clinical and Molecular Implication

The cornerstone of treatment for non-metastatic CRC is the surgical resection of the primary tumor. However, following surgical resection, there is a considerable risk for tumor recurrence in patients with stage III and high-risk stage II CRC. In the absence of post-operative or adjuvant therapy, about 50% of such patients who undergo potentially curative surgery ultimately relapse and die of metastatic disease. The introduction of 5-fluorouracil (5-FU) over 50 years ago, as adjuvant therapy for CRC, has been used to diminish the risk of metastasis. Adjuvant chemotherapy has since evolved to 5-FU in combination with leucovorin and oxaliplatin (FOLFOX), a regimen that is associated with a higher 5-year disease-free and overall survival compared with 5-FU alone in stage III CRC patients. In addition, FOLFOX has been shown to significantly reduce recurrence rates and increase overall survival in high-risk stage II CRC patients [36-37].

In patients with stage IV or metastatic CRC (mCRC), treatment goals are mainly palliative and the 5-year survival rate is less than 10% [14]. With 5-FU adjuvant treatment, overall survival has been shown to be around 12 months. However, the addition of cytotoxic drugs such as irinotecan and oxaliplatin with 5-FU and leucovorin, has significantly improved overall survival to about 20 months [38].

The most important development in molecular markers for metastatic colorectal cancer has been the validation of KRAS mutation status as predictive of non-response to EGFR targeted drugs [7]. However, not all 60% of patients with wild-type KRAS will respond to treatment. Additional factors, such as amphiregulin and epiregulin, might contribute to treatment response; and mutation of BRAF or NRAS, or loss of PTEN or PIK3CA activation might contribute to resistance to EGFR-targeted monoclonal antibodies [39].

BRAF is the main downstream effector of KRAS. Mutations that activate BRAF arise in 8–10% of metastatic colorectal cancers and are mutually exclusive of KRAS mutations. BRAF mutation was associated with poor prognosis (reduced progression-free and overall survival) in patients with metastatic colorectal cancer given anti-EGFR antibodies [40].

Recent studies have suggested an association between loss of heterozygosity at chromosome 18q with poor prognosis in patients with colorectal cancer [41]. Allelic imbalance at 18q had a negative effect on overall survival in patients with stage III microsatellite stable CRCs, but this result was not replicated in other studies [41-44].

Microsatellite instability (MSI) is the molecular fingerprint of the deficient mismatch repair (MMR) system, which characterizes 15% of colorectal cancers. MSI develops as a result of germline mutations in MMR genes or, more commonly, from epigenetic silencing of MLH1 in sporadic tumors occurring in a background of methylation of CpG islands in gene promoter regions and in tumors that frequently show hotspot mutations in the BRAF oncogene [45] (**Figure 6**).

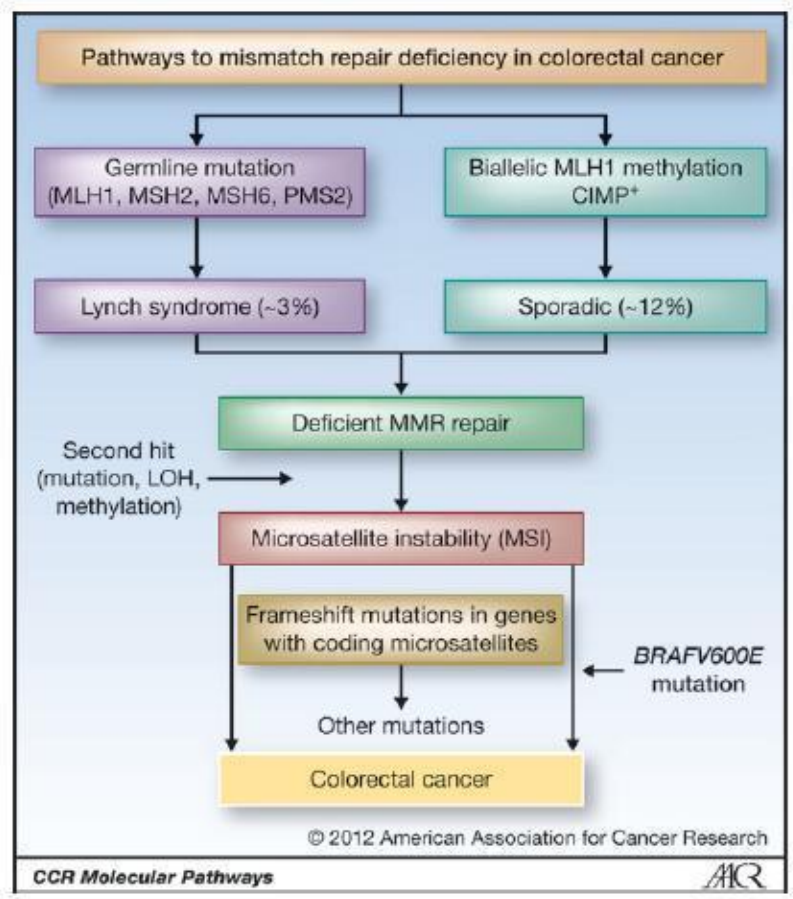

Figure 6 Schematic pathways to mismatch repair deficiency in colorectal cancer. (Microsatellite Instability in Colorectal Cancer: Prognostic, Predictive, and Therapeutic Implications, Clinical Cancer Research, 2012)

Germline MMR mutations give rise to Lynch syndrome or HNPCC, an autosomal dominant disorder that accounts for 3% of all CRC.

Patients with HNPCC develop CRC at a younger age than the general population. They mainly develop proximal colon cancers (70-85% of colon cancers are right sided), and they present a higher risk for synchronous CRCs [46]. Moreover, patients show a higher risk of developing extra-colonic tumors including endometrial, ovarian, gastric, small bowel, pancreatic, hepatobiliary, skin, brain, and urethral tumors. The cumulative lifetime risk of an extra-colonic malignancy in females and males is 47% and 27%, respectively [47]. Histologically, CRCs are often poorly differentiated, mucinous, and have large numbers of tumor-infiltrating lymphocytes [48].

In clinical diagnosis several tool are available to help clinicians to identify patients at risk of HNPCC, including analyses of family histories, tumor testing, mutation prediction models and genetic testing. It is important to obtain a detailed personal and family history. Specific factors indicate patients that have a high-risk CRC condition and should be referred for genetic counseling. The Amsterdam criteria I (**Table II**) were originally developed for research purposes to identify families likely to have Lynch syndrome. More than 50% of families with Lynch syndrome, however, fail to meet these criteria. To increase sensitivity, the Amsterdam criteria II and the Bethesda guidelines were developed. The Amsterdam criteria and revised Bethesda guidelines are used in clinical practice to identify individuals at risk for Lynch syndrome who require further evaluation [48]. One approach to identify patients with Lynch syndrome is to perform genetic testing when individuals meet the personal and/or family history criteria. Because germline mutations in hMLH1 and hMSH2 account for the majority of cases, genetic testing typically starts with analysis of these genes. Limitations of this strategy include high costs and a reduced sensitivity compared to other diagnostic approaches. A second approach to identify Lynch syndrome, which has shown to be cost-effective, is to perform tumor testing when any of the Bethesda guidelines are identified. Various tumor testing strategies exist, most of which begin with MSI and/or immunohistochemistry (IHC) analysis of colorectal tumors [48]. Today, the panel for identification of MSI consist of five mononucleotide repeat markers (BAT25, BAT26, NR21, NR24, and NR27) (**figure 7**). This panel allows accurate evaluation of tumor MSI status of DNA with 100% sensitivity and specificity without the need to match normal DNA [49]. MSI can be classified in MSI-Low defines by instability at only one of the five reference markers, while, MSI-High is defined by instability of at least two markers. Although CRC classified as MSI-Low were phenotypically indistinguishable from microsatellite stable (MSS) CRC, defined by the absence of

allelic shifts in any of the five markers, this terminology is still in use by some authors [50]. Tumor testing with IHC utilizes four antibodies specific for MLH1, MSH2, MSH6, and PMS2 proteins to evaluate tumors for MMR deficiency. The sensitivity of IHC is comparable to that of MSI analysis. However, IHC analysis can direct genetic testing to the appropriate MMR gene when loss of MMR protein expression is identified [48]. Additional tumor testing, including BRAF mutation and hMLH1 promoter methylation analyses, can be helpful in differentiating sporadic vs Lynch syndrome-associated CRCs. Finally, a number of models have recently been developed to help facilitate the diagnosis of Lynch syndrome, including but not limited to PREMM, MMRpro, and MMRpredict. These models utilize personal and family history to estimate the probability that an individual carries a MMR gene mutation [48].

Amsterdam Criteria I and II (AC-I and II) and Bethesda Guidelines

AC-I

• At least three relatives with histologically verified colorectal cancer:

1. One is a first-degree relative of the other two;
2. At least two successive generations affected;
3. At least one of the relatives with colorectal cancer diagnosed at <50 years of age;
4. Familial adenomatous polyposis (FAP) has been excluded.

AC-II

• At least three relatives with an hereditary nonpolyposis colorectal cancer (HNPCC) associated cancer [colorectal cancer, endometrial, stomach, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract, and skin (sebaceous tumors):

1. One is a first-degree relative of the other two;
2. At least two successive generations affected;
3. At least one of the syndrome-associated cancers should be diagnosed at <50 years of age;
4. FAP should be excluded in any colorectal cancer cases;
5. Tumors should be verified whenever possible.

Bethesda Guidelines for testing of colorectal tumors for microsatellite instability (MSI)

1. Colorectal cancer diagnosed in a patient who is <50 years of age.
2. Presence of synchronous or metachronous colorectal, or other syndrome-associated tumors [a] regardless of age.
3. Colorectal cancer with microsatellite instability-high (MSI-H)[b] histology[c] diagnosed in a patient who is <60 years of age[d].
4. Colorectal cancer or syndrome-associated tumor [a] diagnosed under age 50 years in at least one first-degree relative[e].
5. Colorectal cancer or syndrome-associated tumora diagnosed at any age in two first- or second-degree relatives [e].

a Syndrome-associated tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter or renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel.

b MSI-H = microsatellite instability-high in tumors refers to changes in two or more of the five National Cancer Institute-recommended panels of microsatellite markers.

c Presence of tumor infiltrating lymphocytes, Crohn disease-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.

d There was no consensus among the Workshop participants on whether to include the age criteria in guideline 3 above; participants voted to keep <60 years of age in the guidelines.

e Criteria 4 and 5 have been reworded to clarify the Revised Bethesda Guidelines.

Table II Criteria used in clinical practice to identify individuals at risk for Lynch syndrome. ***(Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medico legal ramifications, Clinical Genetics, 2009)***

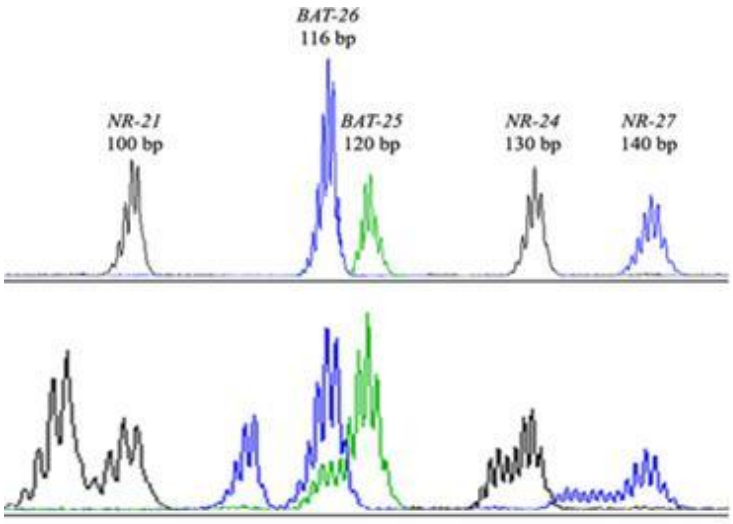

Figure 7 Electropherograms of the analysis of mononucleotide microsatellite markers BAT26, BAT25, NR-21, Nr-24 and NR-27. From top to bottom, an example of MS-Stable CRC, and MSI CRC. (Irrelevance of Microsatellite Instability in the Epidemiology of Sporadic Pancreatic Ductal Adenocarcinoma, Plos One 2012)

Patients with MSI are more common in stage II (~22%) compared to stage III (~12%) CRC, while retain a low prevalence among stage IV CRC (~4%) [51] and numerous studies have been performed to assess the relationship between MSI and MSS CRC-related prognosis. In a retrospective analysis of five randomized clinical trials of patients with stage II and III colon cancer, Ribic et al. [52] demonstrated that patients with MSI-H tumors had greater 5-year overall survival compared with patients with MSS tumors (hazard ratio [HR] = 0.31; 95% confidence interval [CI] = 0.14–0.72, P=0.004). In addition, Popat et al. [53] showed that patients with MSI-H CRC had improved disease-free and overall survival, irrespective of disease stage, compared to patients with MSS CRC, in a meta-analysis of 32 trials. Recently, the PETACC III trial confirmed these retrospective findings by demonstrating prospectively that MSI-H is a strong prognostic factor for relapse-free and overall survival in patients with stage II and III CRC. A subgroup analysis suggested a stronger association of MSI-H with survival among patients with stage II than in stage III CRC. In another recent prospective study, encompassing five randomized clinical trials, Sargent et al. showed that defective MMR (dMMR) or MSI-H was associated with improved disease-free (HR = 0.51; 95% CI = 0.29–0.89, P=0.009) and overall survival (HR = 0.47; 95% CI = 0.26–0.83, P=0.004) compared to proficient MMR (pMMR) or MSS in untreated stage II and III CRC patients [54].

Hence, microsatellite-instability status in addition to be a significant prognostic marker in CRC, might be predictive factor to decide which patients should not be treated with adjuvant fluorouracil [7].

Other common hereditary CRC syndromes are Familial Adenomatous Polyposis (FAP) and MYH-Associated Polyposis (MAP).

- FAP is a highly penetrant autosomal dominant disorder caused by germline mutations of the Adenomatous Polyposis Coli (APC) gene and accounts for less than 1% of all CRC [55-57]. Clinically, patients with FAP present with hundreds to thousands of colorectal adenomatous polyps, usually in the second decade of life. The life time risk of CRC approaches 100% and patients with FAP are also at risk of extra-colonic manifestations such as cutaneous lesions, osteomas, dental anomalies, congenital hypertrophy of the retinal pigment epithelium, desmoid tumors, and extracolonic cancers (liver, pancreas, gastric and small bowel, periampullary, thyroid, and central nervous system) [58]. A distinct variant and less aggressive form of FAP is a Attenuated FAP (AFAP). It is characterized by delayed age of onset and fewer colorectal adenomatous polyps. Extra-colonic manifestations are less common in attenuated FAP [59].
- MAP is characterized by the presence of colorectal adenomatous polyps and an increased risk of CRC. It is an autosomal recessive disorder caused by bi-allelic mutations in the MYH gene [48]. The MYH gene is located on chromosome 1p35 and is a base excision repair (BER) gene primarily targeting oxidative DNA damage [60]. The MAP carcinogenesis pathway appears to be distinct from CIN or MSI. It involves a high frequency of somatic APC mutations, a low frequency of loss of heterozygosity (LOH), and the tumors are usually microsatellite stable [61]. Clinically, patients with MAP have multiple adenomatous polyps, with varying numbers (ranging from 10 to more than 100). CRC develop in about 65% of patients, and usually presents at an older age than classic FAP [62]. One third of patients with MAP could have upper gastrointestinal lesions, but other extra-colonic manifestations are less common than classic FAP [62]. Phenotypically, MAP can be indistinguishable from FAP or attenuated FAP, and therefore genetic testing for MYH mutations should be performed in patients with suspected FAP or attenuated FAP and negative APC germline mutations.

The CIMP pathway provides the epigenetic instability necessary for sporadic cancers to methylate the promoter regions of, and thus epigenetically inactivate the expression of mismatch repair gene, such as, MLH1.

In 1999, Toyota et al. proposed the term CIMP to describe a subset of CRCs that consistently show widespread CpG island hypermethylation at seven different loci defined methylated in tumors (MINT) [63]. Subsequently, methylation at least three MINT loci has strongly been correlated with CDKN2A (p16) and MLH1 methylation constituting the so-called “classic panel” and providing a simplified approach to CIMP definition [64-66]. Using these markers, CIMP positive tumors are more frequently associated with MSI-CRCs than the MSS counterpart and localized to the right colon (up to 40%) than left colon and rectum (3–12%). The CIMP phenotype is, however, uncommon in HNPCC that exhibits MSI, suggesting distinct underlying molecular processes [65-67]. The existence of such a phenotype has largely been debated and a consensus on which markers should be used for its definition has not been reached yet. To overcome this difficulty and support the CIMP phenotype as a distinct CRC molecular trait, Curtin et al. proposed alternative markers (CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1) to the classic list of genes [67]. Using this panel, there is a strong association between CIMP-positive cancers and BRAF mutation, while do not have any relationship with KRAS mutations[66-67].

Currently, CIMP-positive CRCs are defined by a panel of CpG island methylation markers, that are classified as having or not having DNA methylation on the basis of certain thresholds. The CIMP panel of genes and or markers is analogous to the panel of microsatellites used to determine microsatellite status [68]. However, some investigators, rightly advocate for the further refinement of the CIMP-positive group into CIMP-low (or CIMP2) and CIMP-high (or CIMP1) categories [69].

Patients with CIMP positive tumors are characterized by a well-defined cluster of clinic-pathological features, including proximal location and a gender and age bias for the development of CIMP in older women [70-72]. In addition, the CIMP-positive CRCs that are MSI-H share MSI-H characteristics, specifically the relative good prognosis, but in the absence of MSI-H, the CIMP-positive phenotype is characterized by more advanced pathology, poorer clinical outcome and an absence of tumor-infiltrating lymphocytes [72].

Different findings from a large study suggest that CIMP-high is associated with a favorable prognosis in colorectal cancer patients, independently of MSI and BRAF mutation status [73], while studies that examined the predictive utility of CIMP for 5-FU–based therapy were inconclusive [74-75]. In addition, a recent analysis of a population based cohort of patients with stage II and III colon cancers showed that

patients with CIMP-positive tumors did not benefit from adjuvant 5-FU, whereas patients with CIMP negative tumors treated with 5-FU showed improved survival [76]. Of importance, the discrepancies among these studies may be related to the different methylation markers and definitions of CIMP used [63, 68, 73].

Recently, the serrated pathway has been described as a distinct model to colorectal carcinogenesis [77]. It is now evident that what was previously classified as hyperplastic polyps actually encompasses a heterogeneous group of polyps which are now classified as traditional serrated adenomas (TSA), sessile serrated adenomas (SSA) [78]. Many studies suggest that the TSA and SSA progress via different pathways. Indeed, KRAS mutation have been found in almost 80% of TSA, but are very rare in SSA [79], while BRAF mutations and high level of CIMP have been described in 75-83% of SSA [70, 79-81].

Finally, the classification of CRC according to presence of MSI and CIMP has been proposed by Jeremy Jass. Five molecular subtypes, each with a different molecular profile and clinico-pathological features [72].

These are:

- CIMP high/MSI high (12% of CRC); originates in serrated adenomas and is characterized by BRAF mutation and MLH1 methylation.
- CIMP high/MSI low or microsatellite stable (8%); originates in serrated adenomas and is characterized by BRAF mutation and methylation of multiple genes.
- CIMP low/MSI low or microsatellite stable (20%); originates in tubular, tubulovillus, or serrated adenomas and is characterized by chromosomal instability (CIN), KRAS as mutation, and methylguanine methyltransferase (MGMT) methylation.
- CIMP negative/microsatellite stable (57%); originates in traditional adenoma and is characterized by CIN.
- Hereditary Non Polyposis Colorectal Cancer (HNPCC); CIMP negative/MSI high; negative for BRAF mutations.

1.2 Synchronous Colorectal Cancer

The synchronous colorectal cancers (S-CRCs) are defined according to the modified criteria of Moertel [82] as:

- two or more invasive tumors (at least submucosal invasion, pT1);
- lesions clearly separated from each other by at least 5 cm of normal mucosa;
- lesions found in a patient simultaneously or within 6 months of each other (a tumor diagnosed after 6 months can be defined metachronous).

Using the system AJCC / TNM; the most invading lesion (greatest pT) was taken as reference lesion or, called index lesion.

Early diagnosis of S-CRC is important step because, if undetected, will be presented as metachronous tumors, and so, increasing complications related to a second surgical procedure [83-84].

In the literature, S-CRC appears to be preferentially localized in the proximal colon compared to the solitary CRC. Therefore, the recognition of S-CRC as a clinical entity has enhanced the awareness that an accurate perioperative exploration of the entire colon is mandatory in patients undergoing CRC resection.

Although there have been major improvements in techniques available for detecting S-CRC e.g., barium enema, colonoscopy and the more recent CT colonography, the result is still not satisfactory enough.

The barium enema may fail to achieve diagnosis because visualization of the tumour may be obscured by bleeding and inadequate bowel preparation not identifies the 70% of the lesions [85-86]. An annular carcinoma may interfere with cleansing and passing of barium through the lesion to demonstrate a more proximal one. The quality of colonoscopy is better in most cases, because faeces can be removed more easily, but good preparation of the large bowel is still essential. Finally, it has been suggested that all patients should have pre- or postoperative colonoscopy because impalpable synchronous tumors have been reported in up to 34% of resected cases [85]. Nowadays, virtual colonoscopy has become a viable alternative method for the evaluation of the whole colon. CT colonography is above all of value in those patients with stenosis or colon elongation that leads to incomplete colonoscopy. It is not only useful in the evaluation of the proximal bowel, but can also provide surgeons with accurate information about staging and tumor localization [87-88]. Other technical advances, such as magnetic resonance colonography [89] and the combination of CT colonography with PET [90], have been reported as useful tools for the preoperative evaluation of S-CRCs.

These changes in diagnostic modalities may cause a variation in tumor detection and the reported incidence of S-CRCs. This was also suggested by Latournerie et al., who showed an increased use of colonoscopy and an increased incidence of S-CRC in the period 1976 up to 2004 [91]. In fact, since the early 1990s, several guidelines, like those of the American Society of Clinical Oncology (ASCO), recommend a full colonoscopy to ensure a cancer-free and polyp-free colon in the preoperative or perioperative setting in all CRC patients [92].

S-CRC shows a curiosity aspect related to its molecular origin, onset and localization site. In fact these tumors tend strongly to co-localization in the same site, with a concordance rate ranging 67-71% [85, 93, 94], although on the other hand this feature is not confirmed by other studies [95]. However, it is uncertain whether S-CRC is the result of a stochastic oncogenic event or , alternatively, of an increased susceptibility of colonic mucosa to neoplastic transformation, as supported by the established association of S-CRC with metachronous CRC [96].

1.2.1 Epidemiology

In prospective and retrospective studies in patients with sporadic colorectal cancer have established that the frequency of S-CRC is approximately 3.5%. This frequency increases to 10–20% in patients with syndromes of hereditary predisposition to CRC as familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC) and patients with inflammatory bowel diseases (IBD) and ulcerative colitis [85]. HNPCC patients show a high risk (RR = 34) to develop S-CRC and for this reason in 2003 was included in the panel of the revised Bethesda criteria [97]. The incidence figures range from 1 to 7% [98-99], though in earlier studies, the concepts of ‘synchronous’ and ‘metachronous’ had not yet been fully distinguished, and as a result the two were often mixed together in the analysis, causing an overestimation of the synchronous incidence [100].

Several studies analyzed the distribution and survival of the S-CRC in the population, and identify risk factors associated with their occurrence. The main risk factors are represented by gender, age, site and presence of adenomas.

However, S-CRC occurs more frequently in male patients [83-84, 91, 96, 101], although some evidence indicates that there are not significantly differences between men and women [85-86, 94-95, 98, 102-103], and S-CRC occurs in patients of advanced age [83, 91, 94-96, 102], although many studies do not confirm this finding [84-86, 98, 103].

In addition, different authors such as Mulder et al. found a significantly higher risk of having S-CRCs in patients diagnosed with an index tumor in the colon, compared with those located in the rectum, Pinol et al. showed that a proximal localization of the primary CRC was a risk factor for synchronous colorectal neoplasms, although this also included synchronous adenomas and Passman et al. showed that synchronous CRCs (index and second CRC) were more often localized in the right colon, compared with single CRCs [96].

Finally most studies have reported that the incidence of concurrent or presence of adenomas was significantly higher in patients with S-CRCs than in those with solitary cancer. Latournerie et al. reported that 34.1% of patients with S-CRCs had adenomas compared with 19.1% of the single cancers. They also showed that patients with S-CRCs, in which both tumors were in the right colon, were likely to have concurrent adenomas in the right side. Similarly, if both tumors were in the left colon, the adenomas tended to occur in the left colon. Evers et al. also found that in S-CRCs, 52% had adenoma remnants; this result was similar to the findings of Latournerie et al. in a pathological examination, which revealed adenomatous remnants in 24.3% of S-CRCs but only 12.7% of single cancers [100].

Another controversial issue is the expected 5-year survival rates of patients affected by S-CRCs. One study found a higher survival in patients with S-CRCs [104], others have demonstrated that patients with solitary colon primary tumors are more likely to survive [105]. In the first prospective study carried out to eliminate sources of considerable bias that were inevitably present in retrospective case-control studies, Nosho et al. showed that S-CRC cases were significantly associated with poor prognosis. Poor prognosis of S-CRCs is thought to be due mainly to the relatively frequent distant metastasis that occur in synchronous cases. However, even more studies have shown that there is no difference in survival between S-CRCs and solitary CRC when the pathological stages of tumors were identical and the resections were curative [85-86, 91, 95, 106-108].

Nowadays, it is clear that various case-control studies examining characteristic and outcome of patients with S-CRC have provided discrepant results and that, male gender, older age, coexisting adenomas, and worse survival were associated with S-CRC in some series but not in others. All these contradiction likely reflect a bias in selection of index cases as well as in the recruitment of control with solitary CRC. In particular, different series may have variable prevalence of cancers with microsatellite instability, which are associated with multiple lesions but also with better prognosis.

1.2.2 Molecular basis

Regarding the molecular carcinogenesis, CRC represents a heterogeneous disease. S-CRC reflects this intrinsic characteristic, indeed, the occurrence of S-CRC is a natural experiment, which tests whether the molecular events of CRCs are stochastic or if there is a field effect due to unique genetic and/or environmental factors in an individual. S-CRC would be a common risk factor in individuals with inherited predisposition, such as HNPCC, familial adenomatous polyposis (FAP), including attenuated familial adenomatous polyposis (AFAP) and MYH-associated polyposis (MAP). For this reason, the presence of synchronous or metachronous cancers or other HNPCC-associated tumors regardless of age are included in the Bethesda guidelines as an important indicator for MSI testing. On the other hand, the molecular mechanism involved in the onset of sporadic S-CRC, without an apparent familial predisposition, is unclear. In the past, for understand the cause to underlie the multicentricity of cancer in many patients who have multiple tumors in the same organ, Slaughter et al in 1953 introduced the concept of the field effect or field cancerization in the context of oral squamous cell carcinoma. The development of modern molecular technologies has extended the field effect concept by exploring the molecular abnormalities in tissues that appear histologically normal and two popular hypotheses have been proposed. One hypothesis implicates genetic alterations that occur in a stepwise fashion (initiation, promotion, and progression); a clone gains growth advantage and acquires more genetic alterations, which eventually result in cancer. A second hypothesis focuses on epigenetic alterations, which include hypermethylation of the DNA promoter of certain tumor suppressor genes, leading to down-regulation of these genes [109].

In colorectal cancer, the field effect is usually characterized by the simultaneous occurrence of multiple but distinct tumors. In the multistep carcinogenesis model proposed by Fearon and Vogelstein, genetic alterations occur in a stepwise fashion such that a clone with growth advantage proliferates, acquires more genetic alterations, and undergoes another selection for survival and growth, eventually resulting in cancer. According to this model, precancerous cells that are in proximity to cancer cells should have some, but not all, of the genetic alterations that are present in the fully developed cancer. At least three steps are suggested in tumorigenesis: initiation, promotion, and progression [110]. Initiation starts when a loss of specific chromosomal regions occurs, which is frequent in colorectal neoplasia. The common region of loss on chromosome 17p in colorectal tumors has been identified and contains the p53 gene [111]. The second most common region of allelic loss is chromosome 18q, which includes another tumor suppressor gene, Deleted in Colorectal Carcinoma (DCC), encoding a protein with significant

homology to the cell adhesion family of molecules [110]. Other regions, including chromosomes 5q, 1q, 4p, etc., may also be involved. When a stem cell acquires one or more genetic alteration, it forms a patch with genetically altered daughter cells. As a result of subsequent genetic alterations, the stem cell escapes normal growth control, gains growth advantage, and develops into an expanding clone of tumor initiating stem cells or tumor propagating cells. The lesion gradually becomes a field, which displaces the normal epithelium. As the lesion becomes larger, additional genetic hits give rise to various subclones within the field and diverge at a certain time point [112].

The second hypothesis is related to the role of epigenetic. Some studies have established that epigenetic alterations could be indicators of field cancerization in the colon. In support of the role of methylated genes as effectors of the earliest steps in cancer formation, studies of primary human mammary epithelial cells have shown that the aberrant methylation of genes is associated with immortalization as well as with subsequent steps in the malignant progression of these cells [113]. It has also been proposed that the aberrant methylation of genes might lock stem cells in an undifferentiated state, predisposing them to malignant transformation [114]. Shen et al. showed that 50% of tumor-adjacent histologically normal tissue carried detectable methylated MGMT (O6-methylguanine methyltransferase) when the primary tumor had methylated MGMT, whereas only 12% of histologically normal tissue from cancer-free control patients had MGMT promoter methylation. In cases in which the primary tumor did not have methylated MGMT, only 6% of the tumor-adjacent normal tissue carried methylated MGMT [115].

S-CRCs have been shown to have similar epigenetic events through concordant DNA methylation patterns in multiple genes thought to be important in colorectal carcinogenesis [116-117]. A major question related to field cancerization is what underlying factor mediates this process. Although some studies have shown a correlation with folate exposure, most studies have not identified an association with folate exposure and the methylation state of genes in the normal colonic mucosa [118].

Of interest, the methylation state of DNA repair genes, such as MGMT and MLH1, in the adjacent normal mucosa can also be shown to correlate with specific mutations in the tumor DNA, such as KRAS mutations or microsatellite instability, respectively, which are predicted to result from loss of function of these DNA repair genes [119-120]. This finding suggests that epigenetic alterations create a predisposition for specific cancer-related mutations [119-120]. Furthermore, hypermethylation and downregulation of SFRP genes are present in monoclonal aberrant crypt foci lacking APC mutations.

This step is thought to contribute to constitutive WNT ligand signaling and decreased apoptosis, which may cancerize the field and make those cells particularly sensitive to further activating events in the WNT pathway [121]. The methylation patterns of SFRP genes, which regulate the WNT signaling pathway, could be useful for predicting the risk of developing colon cancer [122]. However, the relationship between aberrant gene methylation in the normal colonic mucosa and priming of the mucosa to undergo cancer formation seems to be complex, as there are also data suggesting that the hypomethylation of genes that undergo age-related DNA methylation correlates with the presence of colorectal neoplasms in the CIMP molecular class [123]. At this time, it still remains to be determined whether the methylated genes detected in the normal mucosa are truly indicative of a field effect in the colon or are a marker of an associated phenomenon, such as folate status or tobacco exposure [124]. It is also clear that the methylation state of the right and left colon varies, and that this aspect of normal mucosa sampling needs to be taken into consideration when measuring the methylation state of genes in normal colonic mucosa [125-126].

Recently, one more hypothesis was formulated about the occurrence of S-CRC. This hypothesis is related to the role of serrated neoplastic pathway [77]. In aberrant crypt foci, BRAF activation induces synthesis and secretion of insulin-like growth factor binding protein 7 (IGFBP7), an important mediator of p53-induced senescence. The via CIMP silencing of IGFBP7 and p16INK4a allows the escape from senescence and the progression to SSA. Subsequently, in the sequence SSA to cancer the WNT pathway is implicated. The mechanism of WNT pathway activation is likely to be different from conventional adenomas where APC mutation is common. Methylation-induced silencing of DCC might have an important role in pathogenesis of serrated polyps. DCC suppresses WNT signaling by directly interacting with β-catenin. Silencing of DCC by promoter hypermethylation correlates with BRAF mutation, CIMP, and p16INK4a methylation and it is common in serrated lesions, including hyperplastic polyps, sessile serrated adenomas, and traditional serrated adenomas [77].

Thus, different pathways of colorectal cancer have been proposed to be involved in the occurrence of S-CRC (**figure 8**).

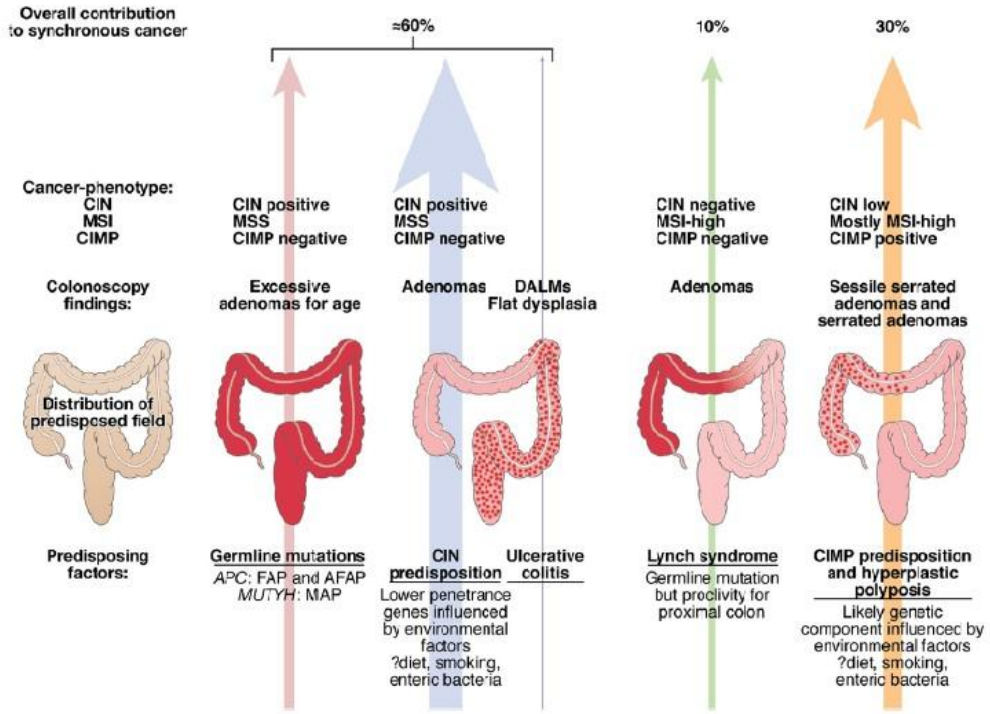

Figure 8 Relative contributions of different pathways of colorectal carcinogenesis to the occurrence of synchronous cancer. Block pink to red shading represents the relative genetic contribution and the red dots depict superimposed patchy environmental events and the typical distribution of pathology. CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylator phenotype; MSS, microsatellite stable; DALM, dysplasia-associated lesion or mass. (Synchronous Colorectal Cancer: Not Just Bad Luck?, Gastroenterology 2009)

2. AIM OF THE WORK

Different studies examining characteristics and outcome of patients with S-CRC have provided discrepant results. Male gender, older age, coexisting adenomas, and worse survival were associated with S-CRC in some series but not in others. These inconsistencies likely reflect a bias in the selection of index cases as well as in the recruitment of controls with solitary CRC. In particular, different series may have variable prevalence of cancers with microsatellite instability, which are associated with multiple lesions but also with a better prognosis.

It is also uncertain whether S-CRC is the result of a stochastic oncogenic event or, alternatively, of an increased susceptibility of the colonic mucosa to neoplastic transformation, as supported by the established association of S-CRC with metachronous CRC. Beside the controversial association of S-CRC with conventional adenomas, evidence also exists that S-CRC might be more frequent among cancers of the serrated neoplastic pathway, which is characterized by a CpG island methylation phenotype, as opposed to tumors of the chromosomal instability pathway.

Given the complex interactions between outcome and distinct molecular profiles, it is obvious that the prognostic significance of S-CRC can be safely assessed only through the analysis of single molecular subgroups of CRC.

To better understand the molecular events occurring in the S-CRC onset and the molecular profiles involved in the prognosis, we underwent retrospective study in a well characterized cohort. These patients performed a perioperative colonoscopy and were classified in molecular subclasses according to microsatellite and *BRAF* status. Finally, we assessed clinico-pathological features and outcome of patients with synchronous advanced neoplasia.

3. MATERIALS AND METHODS

3.1 Study Population and CRC Subgrouping by Synchronous Neoplasia

The study population originally included 1,000 consecutive patients who had undergone resective surgery for CRC at the Humanitas Clinical and Research Center between February 2, 1998 and April 6, 2006. Exclusion criteria were limited to: 1) absence of submucosal invasion at pathology; 2) recurrence of a previously resected colorectal tumor; 3) diagnosis of familial adenomatous polyposis; 4) CRC associated to inflammatory bowel disease. The protocol was approved by the Ethical Committee of the Institution and the informed consent of patients regarding the treatment of their personal data was obtained by the referring physician or by other clinicians involved in the study. At preliminary analysis of records, 119 patients (none of which with S-CRC), were excluded because of incomplete or poor-quality perioperative colonoscopy, this leading to a final study population of 881 subjects.

Demographic and clinico-pathological records were obtained for each patient from the hospital's intranet system. S-CRCs was defined as the simultaneous detection of 2 or more invasive (at least pT1) tumors, separated by at least 5 cm of normal colorectal mucosa, at the time of diagnosis or within 6 months for obstructing tumors. The most invading lesion (greatest pT) was taken as the reference lesion for pathological and molecular classification of S-CRC. By combining macroscopic and histological findings, the following CRC subsets were defined: I) no synchronous neoplasia (n=548); II) synchronous not-advanced adenoma (n=177); III) synchronous advanced adenoma (tubular adenoma 10 mm or greater in diameter, and/or >25% villous component, and/or high-grade dysplasia), (n=106); IV) S-CRC (n=50). To define stage IV disease, pathological reports were combined with surgical findings and with perioperative imaging. The disease-specific survival (DSS) was calculated from diagnosis until death, or until data were censored, as of September 30, 2011. At this date, each patient was confirmed to be alive by direct phone call or by formal inquiry at the local registry of vital statistics.

3.2 Tumor Molecular Subtyping

3.2.1 Assessment of MSI

Tumor samples from all patients were screened for microsatellite instability using the *BAT-26* mononucleotide marker. In patients fulfilling the Amsterdam Criteria II and/or the Bethesda Criteria (n=279), tumor samples were also tested for the *BAT-25* mononucleotide marker. DNA was obtained from paraffin-embedded sections of tumors containing at least 50% tumor cells or from tumor micro-dissections. Tissue sections were deparaffinized for 5 min with 600ul of Xilene and then centrifuged at 13,000rpm for 10 min. Following centrifugation, the Xilene was removed and the tissue pellet was washed with 600ul of 100% ethanol. After a centrifugation at 13,000rpm for 10 min the ethanol was removed and the tissue pellet was putted to dry in termoblock to 80°C for 20 min. Finally, the tissue pellet was suspended with 300ul of proteinase-K digestion to 56°C over-night.

After, *BAT*-25 and *BAT-26* loci were amplified by fluoresceinated primers.

PCR was carried out in 25ul reactions containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphates, 0.4 μM of each primer, 0.5 units of Taq polymerase (Genespin), and 100 ng genomic DNA. The cycles were as follows: 4 min at 94°C, then 35 cycles of 94°C for 15 seconds, 55°C for 20 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Finally, the products were analyzed by capillary gel electrophoresis (ABI PRISM 310 DNA Sequencer, Perkin-Elmer) (**Table III**).

3.2.2 Immunohistochemistry

In all MSI CRC, a defect in mismatch repair (MMR) protein was assessed by the lack of nuclear expression of hMLH1 (clone G-168-15, 1:50, BD Bio sciences), hMSH2 (clone FE11, 1:200, Calbiochem), or hMSH6 (clone 44, 1:200, BD Bio sciences). MMR protein expression was also checked in microsatellite-stable (MSS) tumors from patients fulfilling the Amsterdam Criteria (n=10).

CRC specimens of 3 μm thick sections were cut and processed for immunohistochemistry for MMR protein. After, deparaffining and rehydration, the sections were immersed in an antigen retrieval solution (Diva Decloaker, Biocare Medical) and incubated in the Decloaking Chamber pressure system for 3 min at 125°C and then 5 min at 90°C. Subsequently, two reagents were used Peroxidase-1 and Background Sniper (Biocare Medical) for 10 min. The first was used to quench endogenous peroxidase activity, and the second was used to performed non-specific block. The slides were treated for one hour at room temperature with primary antibodies and then, the MACH 4 Universal HRP-Polymer

(Biocare Medical) was used for 30 min. This detection kit uses a specific probe to detect mouse primary antibodies and is then followed by a horseradish peroxidase polymer (HRP) that binds to the probe. Finally, 3,3-diaminobenzidine tetrahydrochloride (Dako) was used as a chromogen to yield brown reaction products. The nuclei were lightly counterstained with hematoxylin solution (DAKO). MMR protein staining was considered negative when all of the tumor cell nuclei failed to react with the antibody. Adjacent normal tissue served as an internal control for positive staining in almost all tumor tissue sections (>99%).

3.2.3 MLH1, MSH2 and MYH sequencing

Constitutional mutations in MMR genes were searched on DNA extracted from blood lymphocytes and all exons of MYH gene were analyzed in all patients with MSS S-CRC through direct sequencing. Each exon was amplified and sequenced. PCRs were performed in 50µl volumes containing 100 ng genomic DNA, 1X PCR buffer, 1.5mM MgCl2, 0.2 mM each dATP, dCTP and dTTP, 0.2 µM each primer, and 0.5 U Taq Finenzyme (Thermoscientific). PCR products were purified with ExoSap-it (USB® Products, Affymetrics Inc.) following the manufacturer's instructions. In the sequencing reaction cycle, 1µl of purified DNA fragment was blended with each primer (0.1 µM) in a Terminator Ready Reaction Mix containing Big Dye Terminators 1.1 (Applied Biosystems, Foster City, California, USA), denatured at 96°C for 5 min and submitted to 30 cycles at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. A second purification with DyeEx 2.0 Spin Kit (Qiagen) was performed to remove Big Dye. Finally, 10µl of purified single strand DNA were submitted to sequencing analysis on the ABI PRISM 310 Genetic Analyser (Applied Biosystems) (**Table III**).

3.2.4 Multiple ligation probe amplification analysis (MLPA)

Multiple ligation probe amplification analysis (SALSA MLPA P003 MLH1-MSH2 probemix, P248 MLH1-MSH2 probemix, P072 MSH6 probemix, Medical Research Council-Holland, Amsterdam, the Netherland) was performed in mutation negative patients. MLPA reaction was performed following the manufacturer's instructions, characterized by a 4 step protocol: DNA denaturation, Hybridisation reaction, Ligation reaction and PCR reaction. Finally, the products were analyzed by capillary gel electrophoresis (ABI PRISM 310 DNA Sequencer, Perkin-Elmer).

3.2.5 TaqMan SNP Genotyping

All CRC samples were screened for BRAF $^{c.1799T>A}$ mutation by Real-Time PCR using a TaqMan SNP Genotyping Assay (Applied Biosystem). TaqMan MGB probes were designed using the Custom TaqMan Assay Design Tool (Applied Biosystem). The chosen reporter fluorophores were VIC to detect the wild type allele and FAM for the mutant allele (**Figure 9**).

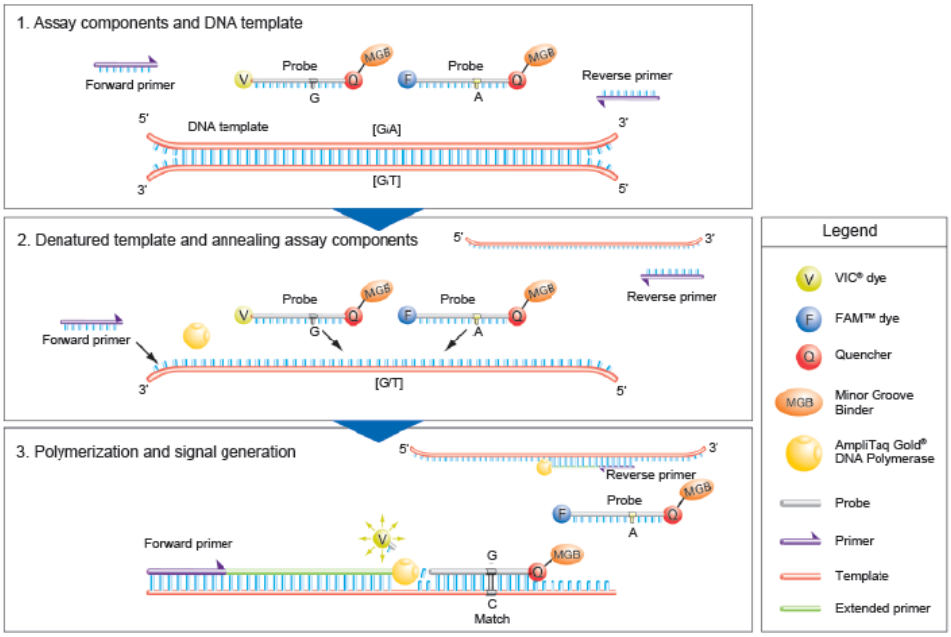

Figure 9 Schematic work-flow of allelic discrimination by the selective annealing of TaqMan® MGB probes.

Table III Primer sequences used for fragment and sequencing analysis

Gene/Exon	Forward Primer	Reverse Primer
BAT 25	GCCTCCAAGAATGTAAGTGGGAg	TGCTTTTGGTTACCACACTTCAA
BAT 26	TGACTACTTTTGACTTCAGCC	AACCATTCAACATTTTTAACCC
MLH1 EXON 1	GAGGTGATTGGCTGAAGGCACTTC	GTGTCCGCGCCATTGAGTGAC
MLH1 EXON 2	GGAGTTTGTTATCATTGCTTGGCTCATA	CTGACTCTTCCATGAAGCGCACAA
MLH1 EXON 3	CCTGGATTAAATCAAGAAAATGGGAATTC	TGACAGACAATGTCATCACAGGAGGAT
MLH1 EXON 4	GTGACAGTGGGTGACCCAGCAGT	ACACTGGTGTTGAGACAGGATTACTCTGAG
MLH1 EXON 5	TGATTTTCTCTTTTCCCCTTGGGATT	ATATCTTGGGACCTCCATTAACTAGTGCAA
MLH1 EXON 6	AGACCTCGCTTTTGCCAGGACA	TTCACCATCTAGCTCAGCAACTGTTCA
MLH1 EXON 7	AAAAGGGGGCTCTGACATCTAGTGTG	TCATGGCTGAGACTGAAACATCATAACC
MLH1 EXON 8	AGGTTATGATGTTTCAGTCTCAGCCATG	CTGTGTATTTGACTAAAGCAAACTCTTAACACAC
MLH1 EXON 9	GGAGGACCTCAAATGGACCAAGTC	GTGGGTGTTTCCTGTGAGTGGATTT
MLH1 EXON 10	AATGTACACCTGTGACCTCACCCCTC	GCATGCTCATCTCTTTCAAAGAGGAGA
MLH1 EXON 11	TACACCATATGTGGGCTTTTTCTCCC	AGGCAAAAATCTGGGCTCTCACG
MLH1 EXON 12.1	TTAGTACTGCTCCATTTGGGGACCTG	TGGACAGGGGTTTGCTCAGAGG
MLH1 EXON 12.2	GGAAGTAGTGATAAGGTCTATGCCCACC	GGCAGAGAGAAGATGCAAGTGATTCA
MLH1 EXON 13	TGCAACCCACAAAATTTGGCTAAGT	TTTCCAAAACCTTGGCAGTTGAGG
MLH1 EXON 14	TGCCTGGTGCTTTGGTCAATGA	TTTTGTGCCTGTGCTCCCTGG
MLH1 EXON 15	CTTCTCCCATTTTGTCCCAACTGG	GTGGAGAGCTACTATTTTCAGAAACGATCA
MLH1 EXON 16	CAGGCTTCATTTGGATGCTCCG	CACCCGGCTGGAAATTTTATTTGA
MLH1 EXON 17	GGGAAAGCACTGGAGAAATGGGAT	TCATTCCAGATCAAAGGGTGGTCATT
MLH1 EXON 18	GTCTGTGATCTCCGTTTAGAATGAGAATGT	ATCTCCTAAAGATTGTATGAGGTCCTGTCC
MLH1 EXON 19	ACATCCCATCAGCCAGGACACC	CACACTTTGATACAACACTTTGTATCGGA

Gene/Exon	Forward Primer	Reverse Primer
MSH2 EXON 1	CAGCTTAGTGGGTGTGGGGTCG	CACTCTCTGAGGCGGGAAAGGAG
MSH2 EXON 2	CAGCATGAAGTCCAGCTAATACAGTGCT	TGCTAATTGCTATTAAAGTGTCTCAAACCA
MSH2 EXON 3	GTTCATAGAGTTTGGATTTTTCCTTTTTGC	GCCTGGAATCTCCTCTATCACTAGACTCA
MSH2 EXON 4	TCATTTTTGCTTTTCTTATTCCTTTTC	TCATTGATACACAGTTTAGGTTTTGAGATA
MSH2 EXON 5	GAGGGACTTCAGAATTTATTTTCATTTTGC	CATTTTTTAACCATTCAACATTTTTAACCC
MSH2 EXON 6	TTGTTCCTCTGTTTTTCATGGCGTAG	TCATGTGGGTAACTGCAGGTTACATAAAAC
MSH2 EXON 7	TGAGCTGATTTAGTTGAGACTTACGTGCTT	TTTATGAGGACAGCACATTGCCAAGT
MSH2 EXON 8	CCTTTTGGATCAAATGATGCTTGTTTATC	CAAACTTTCTTAAAGTGGCCTTTGCTTT
MSH2 EXON 9	TGAAAACAGTAAAATTTAAGTGGGAGGAAA	GAAGTCATCATCTTGGGGACAGGG
MSH2 EXON 10	TTTAGAATTACATTGAAAAATGGTAGTAGG	AAAACTTATCATAGAACATTCACATCATG
MSH2 EXON 11	TTTGGATATGTTTCACGTAGTACACATTGC	CTTCTGTTACCAAAAGCCAGGTGACA
MSH2 EXON 12	TTCCCAAATGGGGGGATTAAATGT	CCACAAAGCCCAAAAACCAGGTT
MSH2 EXON 13	AGCAGAAAGAAGTTTAAAATCTTGCTTTCT	TCTGCAAATATACTTTTCCTTCTCACAGG
MSH2 EXON 14	TGTGGCATATCCTTCCCAATGTATTG	TTCAAGGGTAGTAAGTTTCCCATTACCAAG
MSH2 EXON 15	TGACAAGGTGAGAAGGATAAATTCCATTT	CAACAACAAAAAACCTTCATCTTAGTGTCC
MSH2 EXON 16	ATGAAACAATTTGTCACTGTCTAACATGAC	TATTACCTTCATTCCATTACTGGGATTTT

Gene/Exon	Forward Primer	Reverse Primer
MYH EXON 1	TGAAGGCTACCTCTGGGAAG	GACGTCTGAACGGAAGTTCG
MYH EXON 2	TCATTGTGACTGACTGCTTTG	GGCCCTTAGTAAGTCTCTTAATGT
MYH EXON 3	CTGATGCACAGCCTGTGCA	CCCACTGTCCCTGCTCCTC
MYH EXON 4	CCTCCACCCTAACTCCTCATC	GGTTGGCATGAGGACACTG
MYH EXON 5	GTAGGGGCAGGTCAGCAGT	GAGGCTCTCATCTGGGGTCT
MYH EXON 6	TTGGGGTGGGTGTAGAGAAG	TCACCCGTCAGTCCCTCTAT
MYH EXON 7	ATAGAGGGACTGACGGGTGA	CCAAGACTCCTGGGTTCCTA
MYH EXON 8	CCAGGAGTCTTGGGTGTCTT	CTGGGCACGCACAAAGTG
MYH EXON 9	CAGCCCAGGCTAACTCTTTG	AGCAGAGCTCCTTTGCAGAC
MYH EXON 10	CTGCTTCACAGCAGTGTTCC	GAGGCACAGGGTTGAGTGTC
MYH EXON 11	GTGACTCTGCCCTATGACACTC	AGGTTAGAGGAAGAACTGGAATG
MYH EXON 12	CTAAAGCCCTCTTGGCTTGAGTAG	CACGCCCAGTATCCAGGTA
MYH EXON 13	TAACAAGAGAGAATGGAGGGAATC	AGCCAACATCCTTGGCTATTC
MYH EXON 14	TCCACAGGCCTATTTGAACC	GGAAACACAAGGAAGTACAACAAA
MYH EXON 15	CCCTCACCTCCCTGTCTTCT	TGAAGCCTGGAGTGGAGAAT
MYH EXON 16	GGGAAAGGGAGAGAGGACAA	ACAGGATTCTCAGGGAATGG

3.3 Statistical analysis

Associations between synchronous neoplasia and clinico-pathological or molecular features of the index CRC, were tested using Chi-square test or, if appropriate, Fisher's Exact test for categorical variables and by Student's t-test for continuous variables. Pathological and molecular factors significantly associated with S-CRCs at univariate analysis were entered into a multivariate logistic regression analysis. Survival curves were drawn according to the Kaplan-Meyer method to comparatively evaluate the disease-specific survival of patients with synchronous colorectal neoplasia. To better assess the prognostic role of S-CRCs, as well as of synchronous adenomas, and of tumor MS/BRAF status, Cox proportional-hazard models were also used. For all statistical tests $p < 0.05$ was considered statistically significant.

4. RESULTS

Out of 1,000 patients undergoing resective surgery for newly diagnosed CRC, 50 (5%) resulted to have S-CRC (2 cancers in 47 patients and 3 cancers in 3 patients). At full colonoscopy, 17 of 50 (34.0%) patients with S-CRC and 283 of 831 (34.1%, p>0.5) patients with solitary cancer had at least one distinct concomitant adenoma.

Table IV reports the clinico-pathological and molecular features of the index CRC stratified by the absence or the presence of a synchronous colorectal neoplasia. As compared to patients with no synchronous neoplasia, subjects with synchronous adenoma or cancer were older (66.4+9.9 vs. 63.9+11.8 years; p=0.001), were more frequently men (66.4% vs. 53.3%; p<0.001), and more frequently had a right-sided CRC (41.3% vs. 31.8%; p=0.003). S-CRC was strongly associated with stage-IV disease and with MSI hereditary cancer (HNPCC), but a statistically significant (p=0.04) interaction of the two variables was detected at multivariate analysis. Accordingly, **figure 10** details how only MSS S-CRC were associated with stage IV (p=0.001), whereas MSI CRC presented a low prevalence of metastatic disease even in the presence of synchronous invasive cancer (p=0.88). A full concordance in MS status was observed in all pairs and triplets of S-CRC, except than in one patient carrying a MSI sporadic index CRC and a second MSS cancer. An interaction was also observed between MSI status and BRAF $^{c.1799T>A}$ mutation in determining the association of these two variables with synchronous non-advanced adenoma, whereas no association was detected between BRAF status and synchronous advanced adenomas and S-CRC. **Figure 11** shows that BRAF $^{c.1799T>A}$ mutation was strongly associated with MSI sporadic CRC (37/62, 59.7% vs. 23/787, 2.9% in MSS CRC; p<0.001), and that the prevalence of the mutation was higher in MSI sporadic CRC with synchronous lesions than in those with no concurrent neoplasia (21/26, 80.8% vs.16/36, 44.4%.; p= 0.005). Conversely, in MSS CRC the presence of synchronous colorectal adenomas or cancer was not associated with BRAF$^{c.1799T>A}$ mutation. No MYH germ line mutation was detected in patients with S-CRC. Over a mean post-surgical follow-up of 4.3±2.4 years, a total of 231 CRC-related deaths were registered, 220 (28.0%) among the 787 patients with MSS CRC and only 11 (11.7%) among the 94 patients with MSI cancer (p<0.001). At Kaplan-Meier curves, the presence of S-CRC significantly affected the disease-specific survival of patients with MSS CRC (p<0.001) but not that of patients with MSI cancer (p=0.83) (**Figure 12**).

Table IV. Demographics, Pathology and Molecular Features of CRC with Synchronous Colorectal Neoplasia.

	Synchronous Colorectal Neoplasia							
	None [a]	**Not Advanced Adenoma** [b]		**Advanced Adenoma** [c]		**Invasive Cancer** [d]		
	n=548, ref.	*n=177*	*P**	*n=106*	*P**	*n=50*	*P**	
Age								
years, mean ± SD	63.9±11.8	66.8±9.7	0.003	66.6±10.0	0.02	64.6±10.8	0.70	
Gender								
Male	292 (53.3)	115 (65.0)		77 (72.6)		29 (53.4)		
Female	256 (46.7)	62 (35.0)	0.006	29 (27.4)	<0.001	21 (46.6)	0.52	
Site								
Distal	374 (68.2)	101 (57.1)		64 (60.4)		30 (61.2)		
Proximal	174 (31.8)	76 (42.9)	0.006	42 (39.6)	0.11	19 (38.8)	0.31	
Stage								
I-to-III	419 (76.5)	139 (78.5)		80 (75.5)		30 (60.0)		
IV	129 (23.5)	38 (21.5)	0.57	26 (24.5)	0.83	20 (40.0)	0.01	♦
Histotype								
Adenoca.	506 (92.3)	162 (91.5)		100 (94.3)		48 (96.0)		
Variant	42 (7.7)	15 (8.5)	0.73	6 (5.7)	0.47	2 (4.0)	0.34	
Grade [e]								
G1-G2	406 (79.3)	143 (83.6)		78 (77.2)		38 (77.6)		
G3	106 (20.7)	28 (16.4)	0.22	23 (22.8)	0.64	11 (22.4)	0.77	
Vein Invasion								
No	423 (77.2)	140 (79.1)		82 (77.4)		36 (72.0)		
Yes	125 (22.8)	37 (20.9)	0.60	24 (22.6)	0.97	14 (28.0)	0.40	
MS Status								
MSS	495 (90.3)	153 (86.4)		100 (94.3)		39 (78.0)		
MSI-Sporadic	36 (6.6)	19 (10.7)	0.04	5 (4.7)	0.44	2 (4.0)	0.64	
HNPCC	17 (3.1)	5 (2.8)	0.92	1 (0.9)	0.20	9 (18.0)	<0.001	♦
BRAF								
BRAF WT	516 (94.2)	158 (89.3)		100 (94.3)		47 (94.0)		
BRAF $^{c.1799T>A}$	32 (5.8)	19 (10.7)	0.02	6 (5.7)	0.94	3 (6.0)	0.96	

a “no adenoma”
b “low-grade-dysplasia < 10mm tubular adenoma
c 95 patients with low-grade-dysplasia adenoma (10 mm or greater in diameter or with villous component greater than 25%), and 59 with high-grade-dysplasia adenoma
d Pathological and molecular characteristics of the most advanced cancer (“index” lesion, by pT) were to be inserted.
Of 23 pairs with identical pT (no “index lesion assessable), 22 had fully concordant pathological and molecular features, whereas 1 pair with discordant tumor site was excluded from the analysis of this variable e not assessed in 48 cases (34, 6, 7, and 1 in the four subclasses, respectively) * at Fisher’s exact test
Interactions at multivariate analysis (logistic regression):
♦ “Stage” * “MS status” , p=0.03
● “BRAF status” * MS-status” (excluding HNPCC), p=0.04

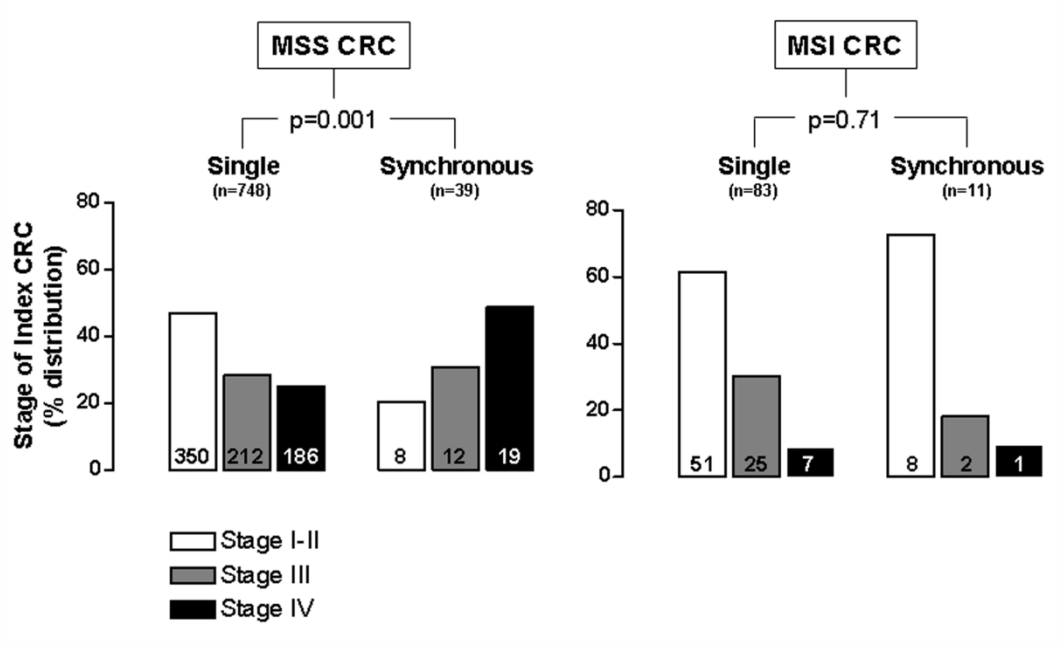

Figure 10 ***Interaction of CRC microsatellite-status and of synchronous colorectal malignancy in determining the association of these two variables with TNM stage. Stage-distribution of synchronous CRC is compared to that of single CRC, stratifying by microsatellite-status. Stage IV was significantly associated with synchronous CRC in patients with microsatellite-stable (MSS) cancer (19/39, 48.7% vs.186/748, 24.9%; p=0.001). The frequency of stage IV disease was not different in MSS CRC patients with no concomitant adenoma (124/495, 25.1%, ref.), with synchronous not advanced adenoma (36/153, 23.5%; p=0.70), and with synchronous advanced adenoma (26/100; 26.0%; p=0.84). The frequency of stage IV disease, in patients with MSI CRC, was not associated with S-CRC (7/83, 8.4% vs. 1/11, 9.1%; p=0.88).***

**** p values at Chi-square or Fisher's exact test, as appropriate (stage IV vs. others)***

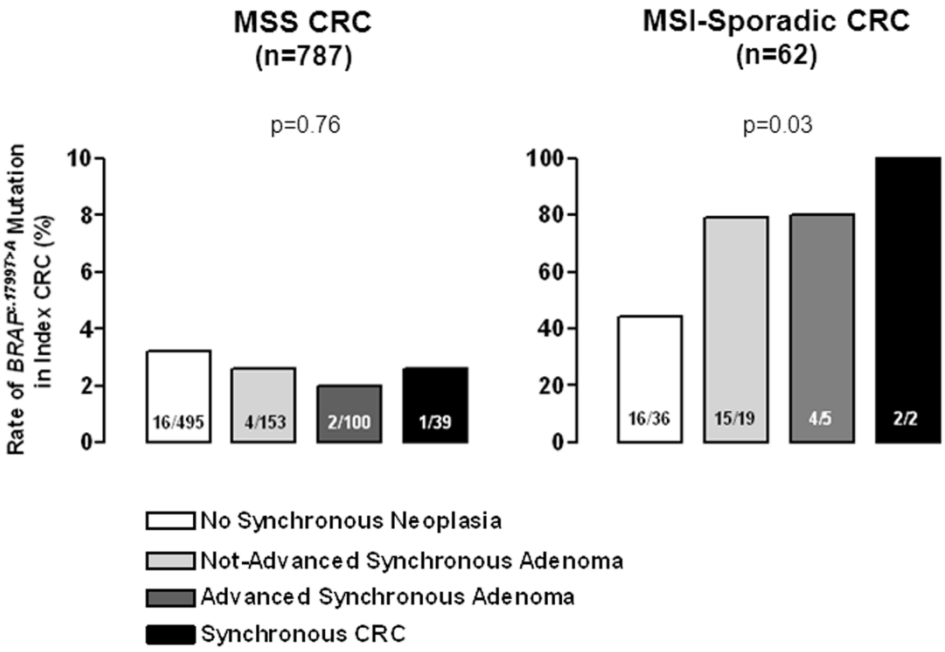

Figure 11 Rate of BRAF $^{c.1799T>A}$ mutation in microsatellite-stable (MSS) and –unstable (MSI) sporadic CRC, by synchronous colorectal neoplasia. BRAF $^{c.1799T>A}$ mutation was significantly (p<0.001) more frequent in MSI-Sporadic (37/62, 59.7%) than in MSS CRC (23/787, 2.9%). In MSS CRC, no association was found between the occurrence of BRAF mutation in the index CRC and the presence of synchronous neoplasia. Conversely, the mutation in MSI-sporadic CRC was less frequent in the absence of synchronous neoplasia (16/36, 44.4%) than in a) any synchronous neoplasia (21/26, 80.8%, p=0.005), b) synchronous not-advanced adenoma (15/19, 78.9%, p=0.01), c) synchronous advanced adenoma or CRC (6/7, 85.7%, p=0.05). HNPCC, which invariably carry no BRAF mutation, were excluded from analysis. P values are from Chi-square or Fisher's exact test, as appropriate .

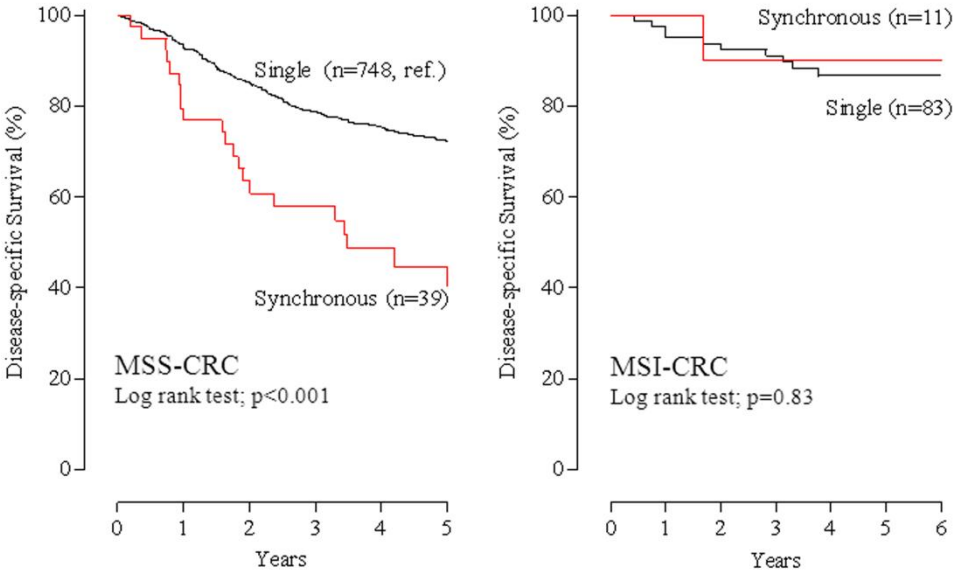

Figure 12 Disease-specific survival of patients with CRC by MS status and by synchronous invasive cancer. Synchronous CRC significantly affected disease-specific survival of patients with microsatellite-stable (MSS) cancer but not of those with microsatellite-unstable (MSI) tumor (Kaplan-Meier curves, Log-rank test).

MSS CRC had a poorer prognosis also in the presence of a synchronous advanced adenoma (p=0.02), but not in the presence of a not-advanced adenoma (p=0.29) (**Figure 13**). The negative prognostic effect of S-CRC or synchronous advanced adenoma was limited to MSS cancers with no BRAF $^{c.1799T>A}$ mutation (p<0.001), whereas BRAF-mutated MSS CRC had a much poorer outcome independently of the presence of a synchronous advanced neoplasia (p=0.98) (**Figure 14**). At Cox proportional hazard models (**Table V**), the presence of synchronous advanced neoplasia was confirmed to be associated with a worse outcome of MSS CRC (S-CRC: HR 2.66; 95% CI, 1.69-4.19; p<0.001; synchronous advanced adenoma: HR 1.59; 95% CI, 1.11-2.26; p=0.01).

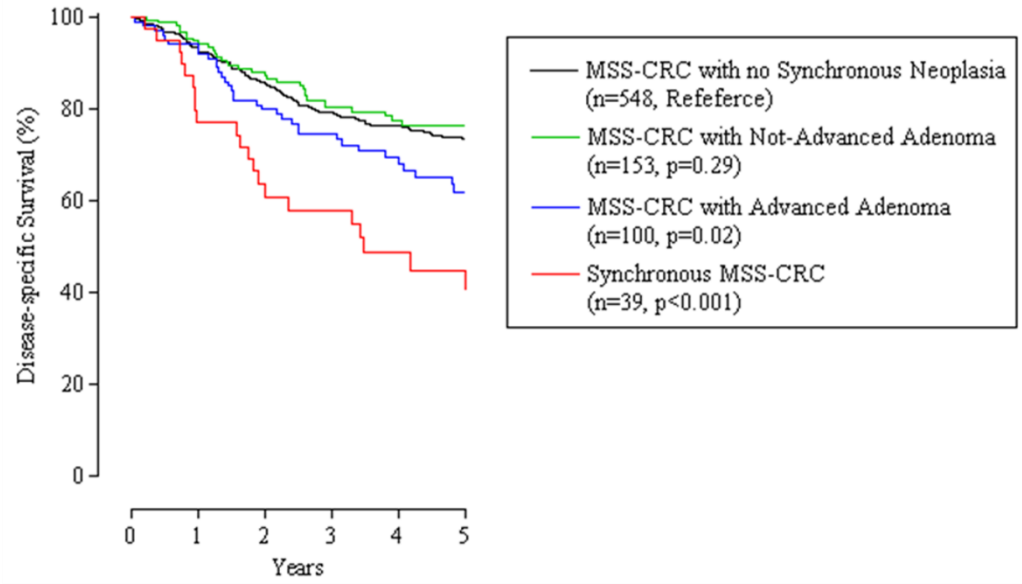

Figure 13 Disease-specific survival of patients with microsatellite-stable (MSS) CRC stratified by synchronous colorectal neoplasia. The presence of synchronous advanced neoplasia, but not that of synchronous not advanced adenoma, negatively affected the survival of CRC patients, (Kaplan-Meier curves, Log-rank test).

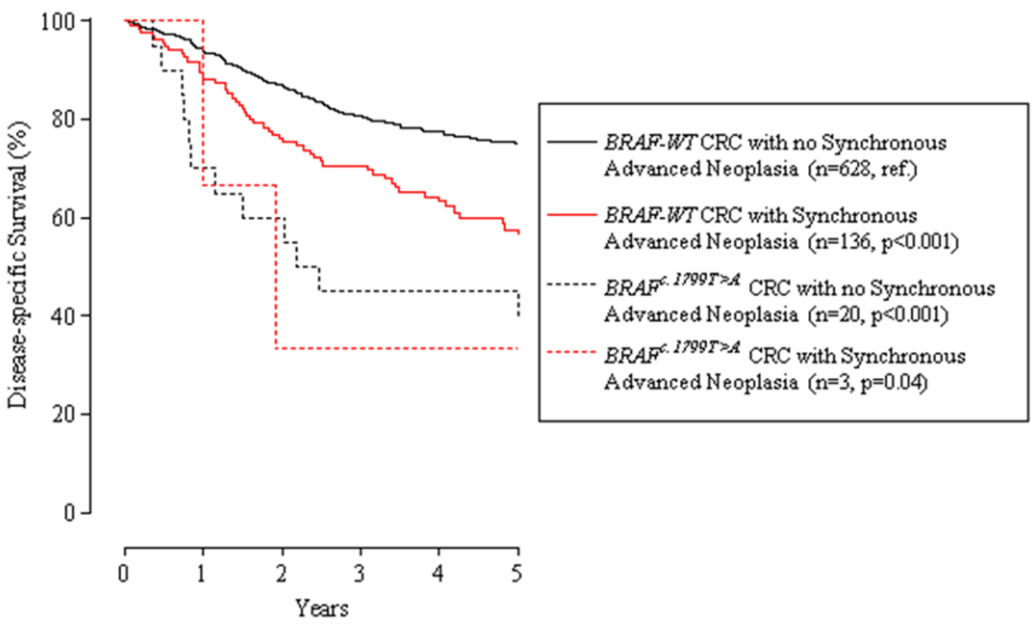

Figure 14 Disease-specific survival of patients with microsatellite-stable (MSS) CRC stratified by tumor BRAF-status and by synchronous advanced colorectal neoplasia. The presence of synchronous advanced neoplasia negatively affected the survival of patients with BRAF-WT CRC, whereas patients with BRAF-mutated CRC had a poorer survival regardless of rarely accompanying synchronous neoplasia (Kaplan-Meier curves, Log-rank test).

Table V Synchronous Advanced Colorectal Neoplasia and BRAF c.1799T>A Mutation as Predictors of Death from CRC (Cox proportional-hazard models).

	Death		Univariate		Stage-adjusted Multivariate	
	No	**Yes**	**HR (95% C.I.)**	**P**	**HR (95% C.I.)**	**P**
A. MSS CRC						
Synchronous Advanced Neoplasia						
None	487	161	1.00 Ref.		1.00 Ref.	
Advanced Adenoma	62	38	1.59 (1.11-2.26)	0.01	1.81 (1.27-2.58)	0.001
Invasive Cancer	18	21	2.66 (1.69-4.19)	< 0.001	1.82 (1.15-2.87)	0.01
***BRAF*-Status**						
WT	558	206	1.00 Ref.		1.00 Ref.	
BRAF $^{c.1799T>A}$	9	14	3.29 (1.91-5.70)	< 0.001	2.16 (1.25-3.73)	0.01
B. MSI CRC						
Synchronous Advanced Neoplasia						
None	67	10	1.00 Ref.			
Advanced Adenoma	6	0	NA	0.45		
Invasive Cancer	10	1	0.74 (0.09-5.75)	0.77		
***BRAF*-Status by Sporadic/HNPCC ***						
BRAF WT - Sporadic CRC	23	2	1.00 Ref.			
BRAF WT - HNPCC	30	2	0.75 (0.11-5.33)	0.77		
BRAF $^{c.1799T>A}$ - Sporadic CRC	30	7	2.68 (0.55-12.9)	0.22		

HR, Hazard ratios < 1.00 represent a decreased risk of death. whereas HR >1.00 represent an increased risk of death
NA, not applicable
* no HNPCC exhibited the BRAF $^{c.1799T>A}$ mutation

Given the higher prevalence of stage IV disease in patients with MSS S-CRC but not in those with synchronous advanced adenoma (see **Figure 10** and its legend), the incremental risk of death associated with synchronous advanced adenoma (HR 1.81; 95% CI, 1.27-2.58; p=0.001) and that conferred by the presence of synchronous invasive cancer (HR 1.82; 95% CI, 1.15-2.87; p=0.01) were almost identical at stage-adjusted multivariate analysis. The occurrence of BRAF $^{c.1799T>A}$ mutation in the index cancer also predicted a higher risk of death from MSS CRC, independently of the presence of a synchronous advanced neoplasia and of TNM stage (HR 2.16; 95%CI 1.25-3.73; p=0.01). On the contrary, neither the presence of synchronous advanced neoplasia nor the BRAF-status of the tumor significantly affected the disease-specific survival of patients with MSI CRC.

5. DISCUSSION

In this large, hospital-based study, patients with MSS CRC had a significantly poorer outcome if originally diagnosed with a synchronous invasive cancer (S-CRC) or even with a synchronous advanced adenoma. The finding is important in that it contributes to a highly controversial issue generated by the fact that most studies failed to recognize any association between S-CRC and poor prognosis [85-86, 91, 95], whereas the only prospective study reported a higher mortality in patients with multiple primary cancers [94]. Notably, our study is unique in having investigated the prognostic role of S-CRC in molecularly defined subgroups of CRC, so to avoid the confounding effect of MSI cancers which more likely develop synchronous malignancies but also have an overall better prognosis. In addition, BRAF $^{c.1799T>A}$ mutation, which is an established marker of CpG island methylation and of poor prognosis [68, 127], was not associated with MSS S-CRC, indicating that neither the occurrence nor the outcome of chromosomal-unstable synchronous cancers are likely due to an epigenetic field effect. Several studies have documented the association between MSI and S-CRC [94, 102, 128]. The strong concordance in MSI status among synchronous cancers also led to the concept that, for genetic and/or environmental reasons, some individuals may be prone to develop multiple cancers through the genetic pathway of microsatellite instability secondary to widespread CpG island methylation and to silencing of the mismatch repair gene MMR MLH1 [28]. This concept was mainly based on the assumption that the majority of MSI S-CRC were sporadic tumors, as suggested by the typically old age of patients with synchronous colorectal malignancies and by the established association between MSI-sporadic tumors and older age [94, 129]. As a matter of fact, no previous study addressing the issue of synchronous cancers, systematically screened patients with MSI S-CCR for germ-line mutations in MMR genes. Therefore, it was a novel, and somehow unexpected finding of our series to see that HNPCC largely accounted for MSI S-CRC (9 of 11, 82%) and that about 1/5 of all S-CRC were diagnosed in patients with hereditary cancer. Consistently, BRAF $^{c.1799T>A}$ mutation was associated only with sporadic MSI CRC, while no mutation was detected in any HNPCC. Overall, data cannot exclude the existence of an epigenetic field effect favoring the development of multiple neoplasia in patients with sporadic MSI CRC, but certainly contradict the idea that this mechanism may account for most synchronous MSI cancers. Rather, our results confirm the appropriateness of the Bethesda criteria which recommend MSI testing of CRC in the presence of multiple primary tumors [130].

The interaction between MS status and advanced stage in their association with S-CRC indicated the need to analyze separately the prognosis of MSI and MSS S-CRC. The analysis revealed that the prognosis of MSI cancers was not affected by any concurrent neoplasia, whereas MSS CRS had a significantly poorer outcome if S-CRC, or even a synchronous advanced adenoma, had been diagnosed. Interestingly, at stage-adjusted analysis, the negative prognostic effect of S-CRC equaled that of synchronous advanced adenoma, indicating that the worsened prognosis of S-CRC likely reflects a more aggressive biological behavior shared by pre-invasive synchronous lesions, rather than a larger cancer burden. This concept is consistent with the well recognized value of S-CRC, as well as of synchronous advanced adenomas, in predicting the future development of metachronous colorectal neoplasia [131-132]. Of note, the presence of synchronous advanced neoplasia and the rare BRAF $^{c.1799T>A}$ mutation were both independent predictors of poor prognosis for MSS CRC. Thus, the hypothesis that an epigenetic field defect may predispose to synchronous neoplasia is plausible for sporadic MSI S-CRC but not for MSS tumors which contribute the majority of multiple primary colorectal malignancies [129]. In this respect, the study by Nosho et al. may have failed to recognize the existing interactions of S-CRC with MSI and BRAF $^{c.1799T>A}$ mutations due to the small number of tumors fully characterized for the MS/BRAF status [94]. Finally, our study failed to detect any MYH germline mutation, not confirming the previously reported association between homozygous or compound heterozygous mutations and S-CRC [133]. Since Cleary et al. found such MYH mutations in less than 1% of the general population with CRC and in about 6% of patients with S-CRC [133], the discrepancy might still reflect a type II statistical error. Alternatively, we might have been more selective in excluding mild polyposis syndromes from our colonoscopy-based clinical series.

Our study has the intrinsic limitation of being a case-control, retrospective analysis. This may have altered the relative contribution of different molecular and clinical subsets of CRC, but a bias in the selection of controls to S-CRC is unlikely, given the consecutive series and the use of complete colonoscopy as the only criteria for inclusion of patients with solitary CRC. Then, the correlations found between synchronous neoplasia and prognosis in single molecular subsets can hardly be interpreted as the result of selection artifacts. The analysis was also limited by the use of BRAF $^{c.1799T>A}$ mutation as the only marker of DNA methylation. Although the BRAF status is validated as a reliable and reproducible marker of cancers with methylator phenotype [68], we might have missed a few methylated tumors potentially identifiable at analysis of multiple CpG islands.

However, we believe that the lack of association between MSS S-CRC and tumor methylation status in our series cannot be disputed on the basis of this limitation.

In conclusion, this study raises the hypothesis that unidentified molecular features may confer a greater aggressiveness to MSS CRC presenting with the phenotype of multiple advanced lesions. If confirmed in large prospective studies, the association between this subset of chromosomal-unstable cancers and poor prognosis would have important implications for postsurgical endoscopic surveillance and, possibly, for adjuvant therapeutic strategies.

Further studies, are necessary to better understand these unidentified molecular features related to MSS S-CRC and CRC with multiple advanced lesions. Many hypotheses have been formulated, but a clear sequence of molecular events that mark the onset of MSS S-CRC and synchronous advanced neoplasia remain to be defined. Recently, studies of whole genome sequencing open new scenarios on the molecular interpretation of this so complex disease. The study of Palles and colleagues shows that the germline mutations in two genes encoding the DNA polymerase ε and polymerase δ predispose to multiple colorectal adenomas and carcinoma [134]. Taken together, these observations could provide a molecular explanation, at least partially, to the onset of MSS S-CRC and synchronous advanced neoplasia, allowing us to read this kind of disease in a different way both biologically and clinically.

6. BIBLIOGRAPHY

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7 SCIENTIFIC PRODUCTS

Presence of Twist1-Positive Neoplastic Cells in the Stroma of Chromosome-Unstable Colorectal Tumors.

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Irrelevance of microsatellite instability in the epidemiology of sporadic pancreatic ductal adenocarcinoma.

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