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Clinicopathological and molecular features of sporadic early onset colorectal cancers

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ABBREVIATIONS

AFAP - Attenuated Familial Adenomatous Polyposis

APC - Adenomatous Polyposis Coli gene

CIMP - CpG Island Methylator Phenotype

CIN - Chromosomal instability

CRC - Colorectal cancer

DFS - Disease free survival

EOCRC - Early onset colorectal cancer

FAP - Familial Adenomatous Polyposis

FOBT - Faecal Occult Blood Test

HNPCC - Hereditary Non Polyposis Colorectal Cancer

IBD - Inflammatory bowel diseases

IHC - immunohistochemistry

LOH - Loss of Heterozygosity

MAP - MYH-associated polyposis

MMR - Mismatch Repair

MSI - Microsatellite instability

TS - Thymidylate synthase

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1 INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide. Although the incidence is now declining in most western countries by 2–3% annually [1], particularly among individuals aged over 50 years, due to improved adherence to screening programs with early detection of colorectal cancer resulting in reduced overall mortality. However, the modern lifestyle changes in less developed countries has led to an increased incidence of CRC, in populations where this cancer was less frequent, causing a significant impact on public health [2]. Moreover, recent studies suggest an alarming increase in the incidence of early onset colorectal cancers (EOCRC) developing among adults younger than 50 years of age, typically below the screening age [3,4]. However, the clinical significance of these epidemiologic findings has not been clarified and especially why young adults without a predisposing genetic abnormality develop colorectal cancer is still unclear.

1.1 Epidemiology and risk factors

Colon cancer is the second most frequent cancer in developed countries in terms of incidence. Moreover, CRC is the third and fourth cause of death from cancer in women and men, respectively.

Worldwide, more than 1.2 million new cases of colorectal cancer are diagnosed each year, while the number of annual deaths from this cancer is over 600,000 [5]. Significant geographical variations with regard to the incidence of cancer in different countries have

been observed; however, comparisons between countries can be problematic due to differences in the availability of epidemiological data. High incidence is found in northern Europe, North America, Australia and recently in Japan, while a low incidence is reported in Africa and central Asia [1]. Although overall statistically significant decreases in CRC incidence and mortality from 1999 through 2008 for both men and women and in virtually all racial and ethnic groups were reported in North America, in 2014 it was estimated that more than 135.000 people were diagnosed with colorectal cancer and more than 50.000 died as a result of it [6]. Incidence rates have risen in many parts of the world, and increases are most prominent in Asia and Eastern Europe. On the contrary, CRC rates have practically stabilized or decreased in developed countries mainly related to the diffusion of screening programs. In particular, the CRC incidence and mortality rates are decreasing among all age groups over 50 years old, yet rising constantly in young adults for whom screening use is limited and symptoms may go unrecognized [7,8].

In Emilia-Romagna region, the incidence of colon cancer in males and females is 77.2 and 59.1 per 100,000 population, respectively, with a mortality rate of 32.8 and 25.2 [9]. A matter of extreme importance is the steady and progressive increase of the incidence over the last twenty years, related to a general improvement of the socio-economic conditions resulting in a greater dietary caloric intake and obesity in the general population, considered to be among the most important risk factors for the development of these tumours [10].

Outside of screening programs, most CRCs are diagnosed in advanced stages of the disease, when tumour invasion or metastasis have already developed, leading to a global 5-year survival rate of approximately 50%. However, decreased CRC incidence and

mortality rates have been shown by recent trends, due to not only screening efficacy on early detection and prevention of neoplastic and pre-neoplastic lesions, but also to reduced exposure to risk factors and more effective treatment. Moreover, nowadays screening programs assess patient risk stratification, taking into consideration patients' personal, familial and clinical history, in order to identify those at high-risk and to determine the most adequate screening strategy. For instance, subjects with a familial history of CRC have a greater risk of developing CRC, from 2 to 6 times higher than the general population, therefore, requiring a more intensive surveillance (Table 1).

Table 1. *Family History and Individual Risk for Colorectal Cancer (CRC)[7]*

Family history	**Approximate lifetime risk**
No history of CRC or adenoma (%)	6
One second-degree or third-degree relative with CRC	1.5-Fold increase
One first-degree relative with an advanced adenomatous polyp	2-Fold increase
One first-degree relative with colon cancer	2- to 3-Fold increase
Two second-degree relatives with colon cancer	2- to 3-Fold increase
Two first-degree relatives with colon cancer	3- to 4-Fold increase
First-degree relative with CRC diagnosed at <50 y	3- to 4-Fold increase

Up to 4-5% of all CRCs are related to two main hereditary syndromes, the Familial Adenomatous Polyposis (FAP) and the Lynch syndrome or Hereditary Non-Polyposis Colorectal Cancer (HNPCC), which will be described further in a successive paragraph. The presence of an inflammatory bowel disease (Crohn's disease or Ulcerative colitis) is also a risk factor for CRC, especially in the case of panniculitis lasting more than 7 years. In these conditions, CRC can develop from an adenomatous dysplastic area within the

affected colonic mucosa, which needs to undergo surgical resection in case areas with high-grade dysplasia are present.

The relationship between lifestyle, dietary habits and CRC cancer have been investigated; obesity, sedentary lifestyle, smoking, an excessive calorie and alcoholic intake, a high-fat diet and low fibre consumption have all been associated with an increased risk of developing CRC [10]. Furthermore, epidemiological studies have clearly demonstrated a significant correlation between CRC and obesity. In particular, in the last 20 years, there has been a considerable rise of the incidence of obesity worldwide in parallel with an increase of CRC, especially in areas where the incidence was low. Moreover, several prospective studies have shown an increased risk in subjects with a Body Mass Index >30. The molecular pathways of the obesity-colorectal cancer association are multi-factorial, related to several mechanisms such as the insulin-IGF1 axis, the effect of adipokines and inflammation.

1.2 Pathogenesis of colorectal cancer

The CRC carcinogenesis is characterized by two main stages: the "initiation" that represents the transition from normal epithelium to adenoma, and the "promotion" phase that marks the passage from adenoma to carcinoma. Several oncogenes and tumour-suppressor genes are responsible for specific genetic alterations for each of these two phases. Such genetic alterations influence proliferation, differentiation and apoptosis, as well as cell polarization and phenotype of the colonic mucosa, and can be inherited or acquired. The inherited genetic alterations or "germline mutations" are responsible for the

development of hereditary colorectal cancers (FAP, HNPCC). Acquired genetic mutations or "somatic mutations" lead to the development of sporadic tumours, which account for the majority of CRCs.

Three pathogenic pathways are known to lead to the development of CRC, all characterized by a progressive accumulation of mutations: the chromosomal instability pathway (CIN), the Microsatellite Instability (MSI) pathway and the CpG Island Methylator Phenotype (CIMP) pathway [11].

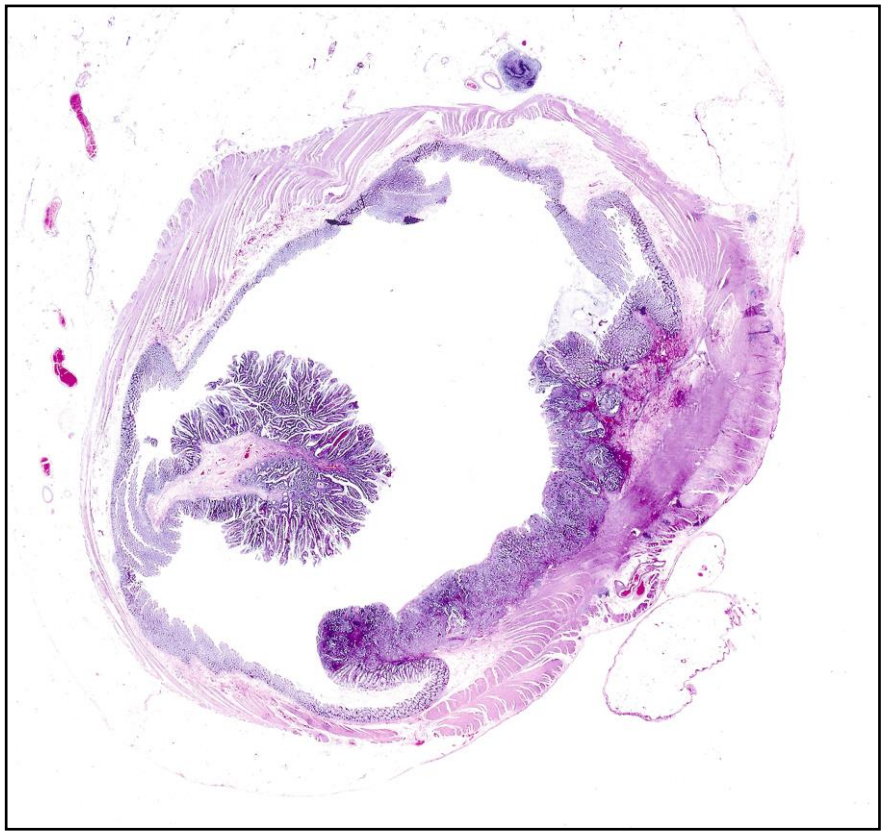
1.2.1 I - The chromosomal instability pathway

Most CRCs occurs sporadically through the chromosomal instability pathway (CIN), associated with the “adenoma-carcinoma sequence” [12], considered the major pathogenic pathway which seems to involve not only adenomatous polyps but also the serrated adenomas (Figure 1). Almost all CRCs arise from adenomas (80% from adenomatous polyps, 20% serrated adenomas) and the adenoma-carcinoma sequence is supported by epidemiological, clinical/pathological and genetic evidence [11]. Indeed, epidemiological studies have shown an association between polyps and carcinomas since populations with a high incidence of colon polyps have an equally high incidence of CRCs. Furthermore, these studies also demonstrated similar prevalence rates between adenomas and CRCs regarding age, population and geographic areas, as well as similar risk factors.

In most cases, the "adenoma-carcinoma" pathogenetic pathway initially involves the APC (Adenomatous Polyposis Coli) gene, a mutation of which is present in 80% of sporadic

cancers. Mutations of the APC gene cause a reduced degradation of beta-catenin leading to its subsequent nuclear accumulation, resulting in an alteration of intercellular contact and abnormal cell proliferation. This first mutation is followed in time by additional genetic alterations such as mutations of the KRAS gene, an oncogene involved in intracellular signalling that is mutated in about 50% of cancers of the colon, Loss of Heterozygosity (LOH) of chromosome 18q21, mutation of the PIK3CA, and deletion of the *TP53* gene found in 70-80% of colonic malignancies. P53 plays the role of guardian of the genome, and its protein regulates basic cell processes, such as cell cycle and apoptosis. Although this sequence of events is observed in the majority of sporadic tumours, the accumulation of various mutations is more important than their specific order of occurrence [13].

Figure 1. *Adenoma of the rectum with associated invasive carcinoma (kindly donated by Prof. Tibor Tot, University of Falun, Sweden)*



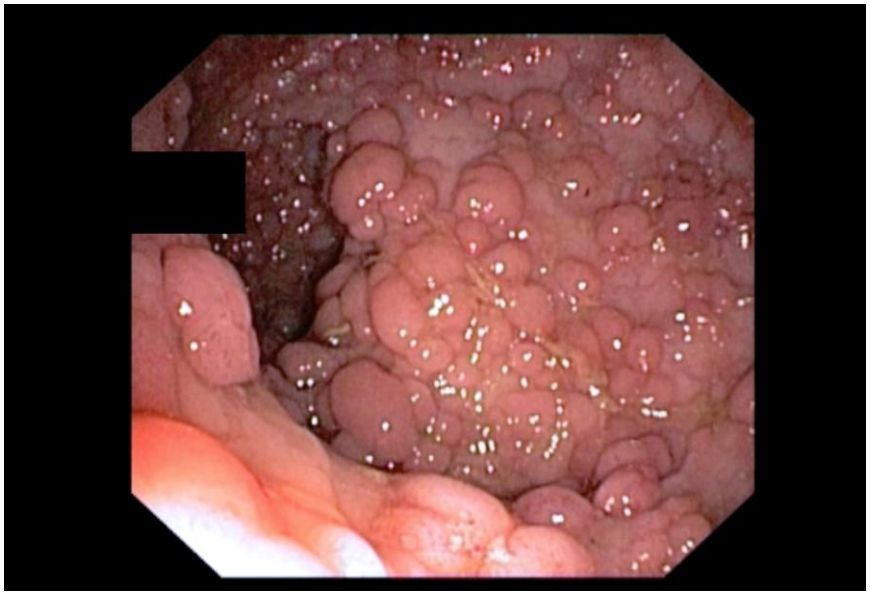
Furthermore, it has been shown that the KRAS gene, when mutated, favours the uncontrolled replication of cancer cells, reducing the sensitivity to chemotherapy treatment, related therefore with a worse prognosis of the disease. In this perspective, the genetic test for KRAS mutations plays an important role in clinical practice, allowing to identify which patients would benefit from oncological treatment [14].

The evolutionary "adenoma-carcinoma" sequence can be found in most colorectal cancers, and this is particularly evident when the tumours develop from an existing epithelial polyp [12]. Most colon polyps are usually single or in some cases, several polyps can be found during the same examination. There are polyposis syndromes in which hundreds or even thousands of polyps develop in the colon and rectum, and often also in other sections of the gastrointestinal tract. The majority of the polyposis are rare hereditary syndromes with an increased risk of developing colorectal tumours. The diagnosis of these syndromes is based on the presence of numerous polyps in the GI tract, mainly in the colon, in more than one member of the same family, most members of which carry specific genetic alterations.

1.2.1.1 **Familial adenomatous polyposis syndrome**

Familial Adenomatous Polyposis (FAP) is a rare autosomal dominant syndrome with high penetrance. Patients with FAP develop CRC in 100% of cases (Figure 2), about 20 years earlier compared to sporadic CRC. FAP is characterized by the development of hundreds to thousands of adenomatous polyps, starting early in life, usually present before the age of 30. Polyps may also develop in other parts of the GI tract, such as glandular polyps of the stomach and adenomas of the duodenum.

Figure 2. *Endoscopic appearance of Familial Adenomatous Polyposis*



An attenuated form of Familial Adenomatous Polyposis (AFAP) is characterized by the development of less than 100 polyps, mainly located in the left colon. In AFAP, the average age for the development of adenomas and CRCs is older compared to subjects with FAP.

Rare variants of FAP are represented by Turcot and Gardner syndromes. The latter is characterized by the association of adenomatous polyps with fibromatosis, epidermal cysts and osteomas, mainly of the skull, the mandible and of the long bones. In the Turcot syndrome, colonic adenomas are associated with tumours of the central nervous system, in particular medulloblastomas.

FAP is caused by a germline mutation of the Adenomatous Polyposis Coli gene, located on the chromosome 5q21, which can be identified by genetic testing. AFAP develops due to a mutation at the 5’ or 3’ extreme of the APC gene or in specific areas of exon 6 [15,16].

The MYH-associated polyposis (MAP), an autosomal recessive condition characterized by the presence of multiple adenomas and CRCs associated with biallelic inactivation of the MYH gene, shows similar clinical aspects to AFAP. Thus, MYH gene mutations should be investigated in all patients with clinical aspects of FAP or AFAP without a mutation in the APC gene. Diagnosis should be performed in early stages of life in order to start endoscopic screening by the age of 10-12 years. If the high number of polyps does not allow an appropriate endoscopic surveillance, prophylactic surgical colectomy is mandatory.

1.2.2 II - The Microsatellite Instability (MSI) pathway

The MSI pathway is involved in approximately in 10-15% of colorectal tumours [17,18]. The microsatellites are highly polymorphic DNA sequences formed by repetitions of single nucleotides (mononucleotides) or pairs of a subunit represented by di, tri, or tetranucleotide. Alterations of the length of these sequences, inside or nearby genes controlling cell proliferation, may modify qualitatively or quantitatively the product of these genes, thus playing a key role in tumorigenesis. The identification of colorectal carcinomas with high microsatellite instability (MSI-H) has also an important clinical significance, because these tumours have specific features such as being most frequently localized in the proximal colon, poor differentiation, mucinous or undifferentiated histology and intense intratumoural and peritumoural lymphocytic infiltrate [19]. Moreover, patients with MSI-H sporadic tumours have a better prognosis and an increased median survival regardless of the stage of the tumour, although these patients have an increased risk of developing metachronous cancer [20].

The identification of colorectal carcinomas with MSI-H is clinically important since these tumours are characterized by more frequent location in the proximal colon, are more resistant to 5-FU chemotherapy but display more frequently a less aggressive behaviour.

The microsatellite instability analysis is performed on a number of specific loci; in particular, the microsatellites panel recommended by the National Cancer Institute include loci BAT25, BAT26, D2S123, D17S250 D5S346e [21,22].

The pathogenetic basis of the microsatellite pathway is due in most cases to a mutation of one of the genes (MLH1, MSH2, MSH6, PMS2 and Epcam) belonging to the group of the Mismatch Repair genes (MMR); these genes are involved in controlling and repairing errors of DNA replication. Their inactivation induces a state of “genomic instability” particularly affecting the microsatellite regions.

In most cases, the tumours with MMR deficiency are predominantly sporadic, caused by epigenetic inactivation of MLH1 by promoter hypermethylation. However, in approximately 20-25% of tumours with microsatellite instability, an inherited mutation (germline mutation) of one of the MMR genes can be found; such genetic alteration is responsible for the development of the Lynch syndrome (or HNPCC: Hereditary Non Polyposis Colorectal Cancer). In addition to the analysis of microsatellite sequences, the use of immunohistochemistry (IHC) with monoclonal antibodies anti-protein MLH1, MSH2, MSH6 and PMS2 has been developed in recent years as a valuable diagnostic test to detect defective expression of MMR genes as genetic mutations for Lynch syndrome [23].

In addition, to distinguish sporadic colorectal tumours with high microsatellite instability, due to methylation of MLH1, from those associated with Lynch syndrome that have a

hereditary mutation in one of the MMR genes, it has been proposed to evaluate the presence of mutations in the BRAF gene. This gene appears in fact mutated in approximately 80-85% of the tumours with MSI and MLH1 methylation, while in cases of HNPCC no mutation occurs, thus, making the analysis of BRAF useful and reliable to identify families with Lynch syndrome [24].

A distinction must be made for those families who meet the clinical criteria for the diagnosis of HNPCC (Amsterdam criteria [25,26]) but which have microsatellite stability and in which no mutation of the MMR genes is found. Various names have been proposed for this variant, such as "Clinical HNPCC" or "Familial Syndrome X". This distinction becomes particularly important as this syndrome shows different clinical characteristics from HNPCC, such as an later onset age and a more frequent localization of the lesions in the distal colon [27,28].

1.2.2.1 **Hereditary Non Polyposis Colorectal Cancer (Lynch syndrome)**

This syndrome is a clinical condition characterized by the onset of colorectal cancer, endometrial cancer and other malignancies, whose development is transmitted in an autosomal dominant or recessive way with a penetrance that can reach 80-90%. This syndrome includes in up to 2-3% of all cancers of the colon which have in most cases an early onset (often to below 45-50 years of age), a localization of the tumour in proximal colon, and a greater risk of developing multiple cancers in the same patient [29].

The "Amsterdam criteria" were developed by the International Collaborative Group on HNPCC to clinically identify cases of Lynch syndrome. To do so, these international

guidelines used several parameters such the young age of cancer onset, the number of colon cancers among members of the same family, the exclusion of FAP and the histopathological confirmation of each tumour. However, once it was discovered that germline mutations of MMR genes and the microsatellite instability were the bases for the development of CRCs, the Bethesda guidelines were developed, providing physicians specific clinical criteria (Table 2) to identify families or patients with Lynch syndrome in whom perform MSI analysis.

Table 2. *The Revised Bethesda Guidelines for testing colorectal tumours for microsatellite instability*

Tumours from individuals should be tested for MSI in the following situations:
Colorectal cancer diagnosed in a patient who is less than 50 years of age.
Presence of synchronous, metachronous colorectal, or other HNPCC-associated tumours,* regardless of age.
Colorectal cancer with the MSI-H histology diagnosed in a patient who is less than 60 years of age.
Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under age 50 years.
Colorectal cancer diagnosed in two or more first- or second-degree relatives with HNPCC-related tumours, regardless of age.

*endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain tumours

Nevertheless, despite meeting the Amsterdam criteria regarding the clinical diagnosis of HNPCC, in some cases families do not show MSI or mutation of the MMR genes. Various names have been proposed for this variant, such as "Clinical HNPCC" or "Familial Syndrome X".

Identifying families with Lynch syndrome allows to implement surveillance and early diagnosis. The "lifetime risk" of colorectal cancer in families with MMR mutation is 28-75% in men and 24-52% in women. In these cases, colonoscopy is recommended every 1 or 2 years starting from 20-22 years of age, in order to reduce the risk of CRC by more than 60% [23].

1.2.3 III – The CpG Islands methylator Phenotype pathway

This pathway is related to the presence of hypermethylation of the promoter of several genes, in particular of tumour suppressors, leading to a consequent silencing of such genes. This pathway is most frequently associated with CRCs developing from serrated adenomas. The CIMP+ colorectal cancers frequently present a mutation of the BRAF gene, member of the RAF kinase family of growth-signal transduction protein kinases, whose deficiency reduces apoptosis.

1.3 Clinical features of colorectal cancers

1.3.1 Sporadic colorectal cancer

The adenocarcinoma is most common type of CRC representing almost 95% of cases. The remaining 5% includes lymphomas, carcinoids and squamous cell carcinomas. In most case series, 55-65% of CRCs develop in the left colon and rectum, whereas multiple carcinomas are found from 3 to 6% of cases [30].

From a macroscopic point of view, right colon cancers tend to grow more frequently as exophytic masses, while tumours from the left colon show usually a depressed form or have an annular shape, affecting the entire circumference of the colon, causing stenosis and obstructive symptoms.

Histological features of adenocarcinomas from the right and left colon are similar, although the mucoid forms are more frequent in the proximal colon. The histological pattern may range from well-formed glandular structures to poorly differentiated irregular masses. Extracellular mucin can be observed in tumour stroma in which the neoplastic cells appear to float; inflammatory or desmoplastic aspects may be particularly evident at the margins of the tumours. Necrosis is usually abundant and cellular mitosis, often abnormal, can be numerous. The neoplastic cells tend to infiltrate nerves, blood vessels and the intestinal wall as far as the serosa. All these parameters are useful for determining the stage of the disease.

CRC symptoms depend on the stage, size and location of the tumour. Indeed, cancers of the caecum and right colon, more frequently lead to microcytic anaemia from chronic blood loss, fatigue, weakness or unexplained weight loss. On the contrary, CRCs that are more distal may cause acute rectal bleeding, abdominal pain and discomfort, and changes in bowel habits. The presence of a palpable mass or of obstructive symptoms develop during the advanced stages of the disease due to the invasive tumour growth. Perforation is a late complication and when it occurs, it is often related with high mortality rates. Other symptoms can develop due to cancer invasion and compression of adjacent structures such as bladder, ureter, vagina, or to local and distant metastases, mainly lymph nodes

and liver. Nevertheless, usually the symptoms appear vague and nonspecific, often indicative of an advanced stage of the disease at the time of the diagnosis.

In the presence of the previously mentioned symptoms, or in case of positive Faecal Occult Blood Test (FOBT), the diagnostic confirmation of the presence of a CRC is generally easily obtainable with a colonoscopy.

The introduction of screening programs has significantly increased the detection of CRCs in early stages, before they develop symptoms, thus reducing both morbidity and mortality. Indeed, the stage of the cancer is most important factor that affect the prognosis. For instance, when the tumour is considered operable, surgery can be curative with a 5-year-overall survival ranging between 55 and 75% [31]. Moreover, for stage I lesions, that are confined to the submucosa and muscularis mucosa, the 5-year survival is greater than 90%, whereas it declines to 70% when the tumour infiltrates beyond the muscularis (stage II). In the presence of lymph node metastases, the survival rate drops to 40-50%, while if lung or liver metastases are present, the 5-year survival is approximately 20% [31].

1.3.2 Early onset colorectal cancers

Approximately, 15% of CRCs occur before the age of 50, mostly related with inflammatory bowel diseases (IBD), hereditary nonpolyposis colon cancer, and polyposis syndromes of the gastrointestinal tract. In this high-risk population, early endoscopic screening has shown to be effective in reducing mortality from colorectal cancer [32]. However, up to 5-7% of colorectal cancers occur in patients with less than 50 years of age (Early Onset

Colorectal cancers - EOCRC) with no evidence of familial predisposition or belonging to hereditary syndromes [33].

During recent decades, a significant rise in CRCs incidence and mortality has been reported in young adults under 50 years of age at diagnosis in many parts of the world [7,34-36], although the aetiology of this increase is currently unknown. Increasing incidence is mostly driven by rising rates of rectal cancer, as seen from 1992–2005 in the United states where young-onset rectal cancer has increased by 3.5% per year in men and 2.9% per year in women [4].

These early-onset neoplasms are located most often at the level of the left colon and present a more aggressive clinical behaviour. Case series of early-onset colorectal neoplasms have been studied and analysed [33,37,38], however, little is known about why young adults without a predisposing genetic abnormality develop colorectal cancer and the pathogenetic pathway that leads to the early onset of these tumours has not yet been clarified.

2 OBJECTIVE AND AIMS OF THE STUDY

The overall objective of the study is to improve diagnosis, treatment and prevention of early onset colorectal cancers, by studying a large population of patients younger than the age of 50, who present no predisposing genetic risk factors at the time of diagnosis.

The specific aims include:

- defining the clinicopathological features and the stage at presentation of sporadic early onset colorectal cancers

- understanding whether the histological, immunohistochemical and molecular analyses associate with particular clinicopathological and oncologic follow-up parameters of sporadic EOCRCs.

- understanding whether the early onset colorectal cancers may be defined as a distinct entity when compared to a consecutive series of sporadic CRCs that underwent surgery, with respect to their clinicopathological, histological and/or molecular features.

3 METHODS

3.1 Patient selection

To identify the cases of early onset colorectal cancer reported between 2006 and 2014, the prospectively maintained databases of the two oncological surgeries were reviewed together with the registries of the pathology department of the Sant'Orsola-Malpighi University Hospital. Only patients younger than the age of 50 were included in the study. Inclusion criteria were as follows: the diagnosis of colorectal adenocarcinoma confirmed on pathology; no presence of other types of colon cancer such as squamous cell carcinomas, carcinoids or lymphomas; no patients that had hereditary nonpolyposis colon cancer, inflammatory bowel disease, polyposis syndrome, or had a known family history for these conditions.

Patients with a positive family history for sporadic colorectal cancer or cancers at sites other than the gastroenterological tract were not excluded from analysis. Altogether, 94 EOCRCs and were found and included in the study.

Moreover, to compare the data of EOCRCs, a consecutive series of 192 sporadic CRCs that underwent surgery in the same Hospital and that had been analysed as reported in a previous study by our research group was reviewed and used as a control group [39].

The medical records from all EOCRC patients were reviewed and the following data was extracted: patient demographics; comorbidities; family history for colorectal or other cancers; alcohol use; smoking; presenting symptoms for the EOCRC group; date of surgery; Tumour-Node-Metastasis (TNM) staging (Table 4); pathologic staging (Table 3); pattern of growth; tumour grade of differentiation: well, moderate, or poor; mucinous

adenocarcinoma: mucinous differentiation of more than 50% of the tumour; location of the tumour defined as right sided (cecum, ascending colon, hepatic flexure, transverse colon) or left sided (splenic flexure, descending colon, sigmoid colon) or rectum. Moreover, the presence of synchronous cancers was taken into consideration when a secondary lesion was found away from the primary tumour at the time of diagnosis.

Table 3. *Colorectal cancer staging systems*

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

Table 4. *TNM classification for colorectal cancer*

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into the pericolorectal tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to 1 organ or site (liver, lung, ovary, nonregional node)
M1b	Metastases in more than 1 organ/site or the peritoneum

Regarding the control group, the following data were available: patient demographics; Tumour-Node-Metastasis (TNM) staging; pathologic staging; pattern of growth; tumour grade of differentiation: well, moderate, or poor; mucinous adenocarcinoma: mucinous differentiation of more than 50% of the tumour; location of the tumour defined as right sided (cecum, ascending colon, hepatic flexure, transverse colon and splenic flexure) or left sided (descending colon, sigmoid colon and rectum).

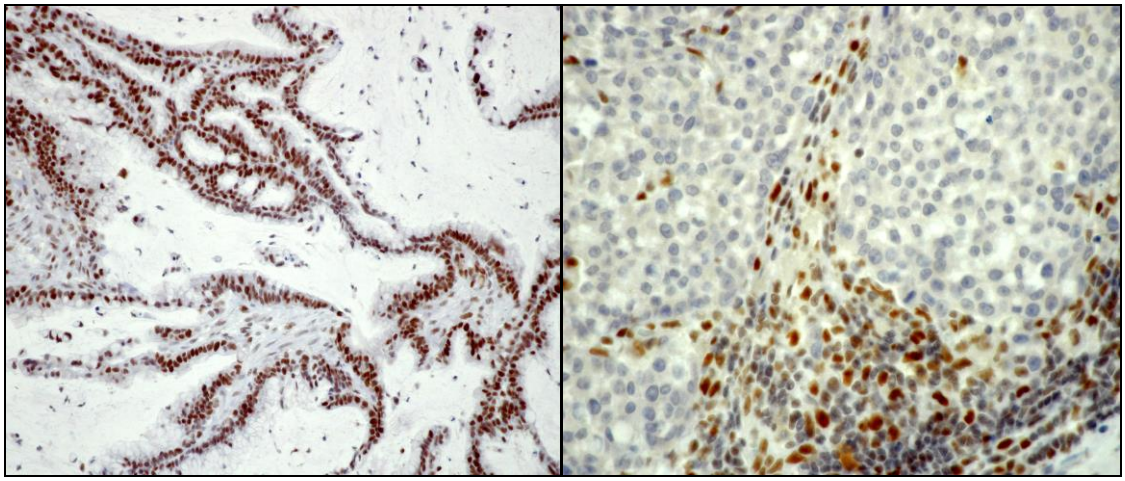
3.2 Molecular analyses

Records from the pathology department were reviewed searching for available molecular data for each included case of EOCRC. In particular, the data on routinely performed analyses was collected, including KRAS and BRAF genotype, MLH1 and MSH2 protein levels, as well as Ki-67, p53 and TS protein levels.

In case of lacking data, if material was available, analysis were completed for the remaining cases, in particular regarding KRAS (codons 12 and 13) and BRAF codon 15 (V600E) mutational analysis, performed using direct sequencing of polymerase chain reaction (PCR)–amplified products [40], and the MMR proteins immunohistochemistry staining (Figure 3). For the latter, the sections containing neoplastic tissue fixed in formalin and embedded in paraffin were immunostained by using the automatic processing immunostainer Benchmark Ultra Company Ventana (Ventana Medical Systems, USA). The processing protocols were as follows: anti-MLH1 clone M1 pre-diluted, Antigen Retrieval (AR) with Ultra CC1 for 32 Min. At 95 ° C, incubation for 16 Min. At 37 ° C, with detection system OptiView DAB + amplification detection kit; Antibody anti-MSH2 clone

G219-1129 pre-diluted, AR with UltraCC1 for 52 min at 95 ° C, incubation for 32 min at 36°C, with detection system UltraView DAB detection kit + Amplification. The whole neoplastic population was evaluated by selecting randomly at least 30 fields at 200x for a minimum number of 5,000 cells. The evaluation was performed by IMAGE-Pro Plus v.5.0.1 software (Media Cybernetics Inc., USA). The final value was expressed as a percentage of the positive neoplastic population. The cut-off used to identify cases with significant loss of expression of MLH1 or MSH2 (High instability) was <= 1%.

Figure 3. *MSH2 immunohistochemical staining*



For the IHC analysis of p53 and TS, sections were stained using monoclonal anti-p53 (clone BP53.12, Novocastra Laboratories, Newcastle-upon-Tyne, England) and anti-TS (clone TS106, Zymed Laboratories) antibodies and following previously described protocols [39]. Cytoplasmic TS immunoreactive population was evaluated according to positive tumour cell percentage and staining intensity as follows: score 0 if <1%, score 1 if >1% <20%, score 2 if >20% <50%, score 3 if >50% <80%, score 4 if >80%; intensity: score 1 (weak), score 2 (moderate), and score 3 (strong). A final score was considered by

adding the two score values as follows: negative (range 0-2), low (3-5), and high (6-7) expression. Nuclear immunostaining of p53 was classified as negative for cut-off values <10% [39].

3.3 Oncological follow-up and outcome

The records of the two Oncology Departments of the University Hospital were queried to extract data regarding oncological treatment and postoperative follow-up of EOCRCs.

In particular, the following data were extracted: use of neoadjuvant therapy, use of adjuvant treatments, treatment duration (number of cycles), treatment side effects, surveillance regimens, development of local and distant recurrence, need for further treatment (oncological or surgical) and tumour-related death. In particular, the date and site of the first tumour recurrence was extracted, and recurrences were categorized as local if they were perianastomotic and as distant if they involved lymph nodes, liver, lung, or other distant organs.

Patients were followed to their date of death or their last contact with the medical centre.

3.4 Statistical analyses

Data were summarized using frequencies and percentages for all categorical variables, and all continuous variables were described as means with standard deviations. Categorical variables were compared using the chi-square test or Fisher's exact test as appropriate, whereas the continuous variables were analysed using two-tailed unpaired t

test, with considering significant the differences for which $p<0.05$. Cancer survival rates were calculated from the date of diagnosis using the Kaplan–Meier method.

Data were entered into Microsoft excel spreadsheet and subsequently imported into Medcalc software for windows, version 16.1, for statistical calculations.

4 RESULTS AND DISCUSSION

4.1 Clinicopathological features of early onset colorectal cancers

Over a 10-year period, between 2005 and 2014, 94 patients with adenocarcinoma of the colon-rectum diagnosed before the age of 50 years and operated at the University hospital Sant'Orsola-Malpighi were included in the study. The distribution according to gender was similar, with males and females representing 54% and 46% of cases, respectively.

The mean age at surgery was 43.2 ± 3.9 years, distributed as follows: 48 were 45-49 years old, 28 were 40-44 years old, 13 were 35-39 years old, 4 were 30-34 years old and 1 was under 30 years of age. Such a distribution in age of EOCRC is in line with data published in previous studies [8].

Demographic and clinical features of all included cases are listed in table 5.

The most common site of primary tumour was the rectum (40%), followed by left colon (32%) and right colon (27%). Figure 4 shows the specific location of EOCRCs in the colorectum. Synchronous malignant lesions were noted in four patients (4.3%).

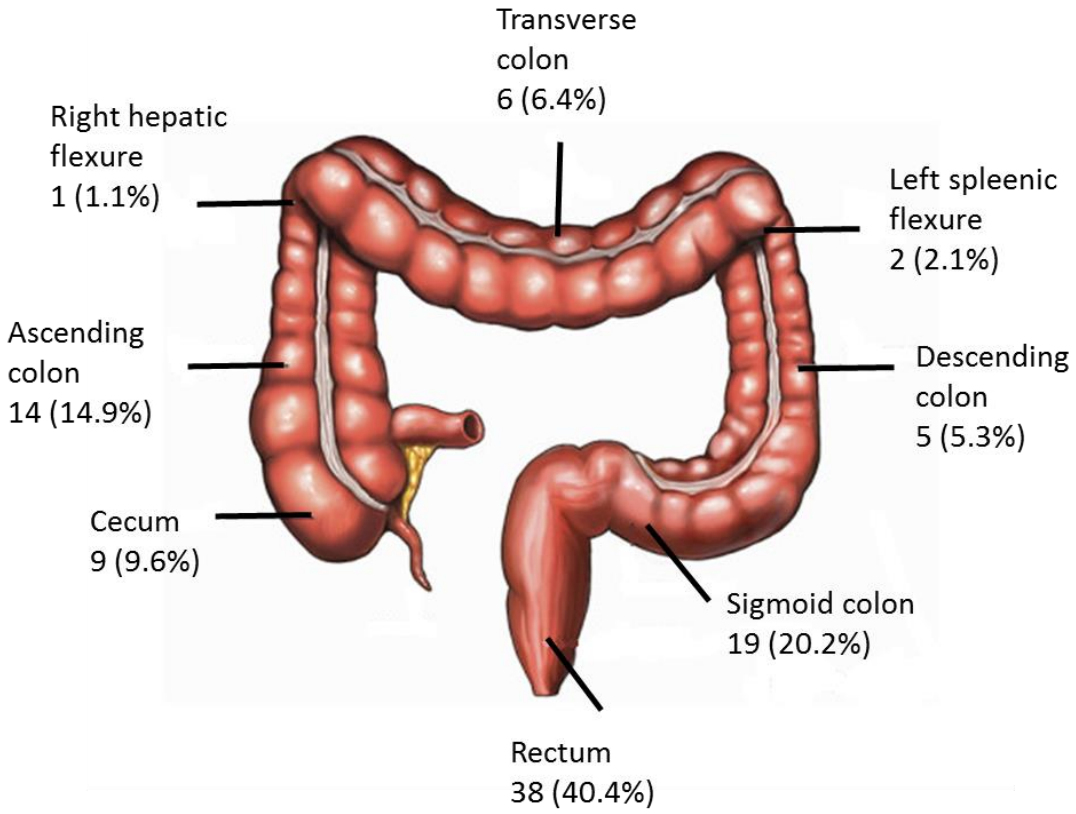
Seventy-eight (83%) patients were symptomatic at the time of diagnosis. The most common presenting symptoms were abdominal pain (34%), haematochezia (22%), rectal bleeding (21%) (Table 6). Of the 16 asymptomatic patients, evaluations were pursued mainly due to finding of anaemia, positive faecal occult blood test abdominal or presence of a mass. Patients with rectal cancer were more likely to present with the symptoms compared to colon cancer, although this tendency was not statistically significant (92% vs. 77%, $p = 0.07$).

Table 5. *Demographic and clinical features of the cohorts*

	EOCRCs (n=94)	***CRC Controls CRCs (n=192)***
Age, years (Average ± SD)	43.2 ± 3.9	67.3 ± 12.1
Sex		
Male	51 (54%)	107 (56%)
Female	43 (46%)	85 (44%)
BMI (Average ± SD)	23.7 ± 3.5	N/A
Alcohol (>35 gr/die)	20 (21.3%)	N/A
Smoking	35 (37.2%)	N/A
Dyslipidaemia	10 (10.5%)	N/A
Location		
RIGHT COLON	30 (32%)	83 (43%)
LEFT COLON	64 (68%)	109 (57%)

Clinical features at presentation in this cohort are similar to those found in other published series on young onset CRC. Indeed, the two most common symptoms at presentation, reported both in systematic reviews and in large cohort studies, are rectal bleeding and abdominal pain, since most studies include haematochezia among the rectal bleeding cases [41,42].

Figure 4. *Specific location of EOCRCs in the colorectum*



Regarding tumour staging, half of EOCRCs showed an advanced stage with either stage III (34%) or stage IV (16%) upon presentation. Moreover, 44% of patients had lymph node localization (N1 or N2) and 12% had already distant metastasis (M1) at presentation considering the TNM staging. The issue of a late diagnosis of EOCRC is well known and is related to the fact that almost all cases are diagnosed due to the presence of symptoms developed during more advanced stages of the disease. Moreover, late diagnosis may also result from the clinician's failure to consider the possibility of malignant disease in the differential diagnosis due to the young age [41].

Table 6. *EOCRCs presenting symptoms at first diagnosis**

Symptoms	***N. of cases***
Abdominal pain	32 (34%)
Haematochezia	21 (22.3%)
Rectal bleeding	20 (21.3%)
Change in bowel habits	16 (17.1%)
Other	12 (12.8%)
Anaemia	10 (10.6%)
Weight loss	5 (5.3%)
Weakness	4 (4.3%)
FOBT positive	4 (4.3%)

* Numbers total more than 100% because subjects were allowed to have multiple presenting symptoms

Histologically, 10% of the cancers were well differentiated, 70% were moderately differentiated, and 20% were poorly differentiated. Mucinous and singlet cell histology was seen in 20 (21%) and 2 (2%) cases, respectively. Finally, the majority of EOCRCs showed an infiltrating tumour growth.

These finding are in accordance with the histological features reported in previous case series of EOCRCs [41].

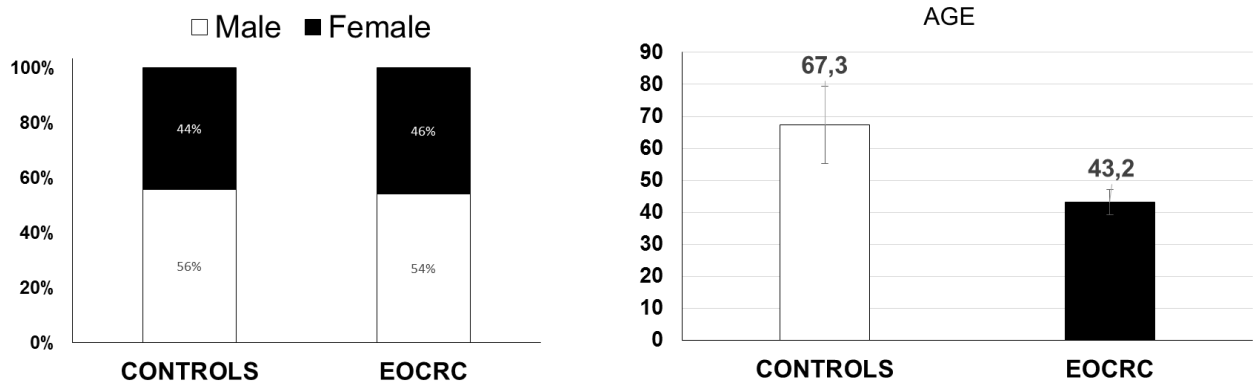
4.2 Comparison between EOCRCs and CRC control group

The control group was composed by 192 consecutive patients surgically resected for sporadic colorectal cancer (thus, not belonging to known HNPCC or FAP families), and collected during the year 2001 at the Policlinico S.Orsola.

Demographic and clinical characteristics of all CRCs are reported in table 5.

The mean age of this group was 67.3 ± 12.1 (Figure 5B). Male gender represented 56% of cases, similar to the EOCRCs group, indicating that there is no gender bias for development of EOCRC (Figure 5A).

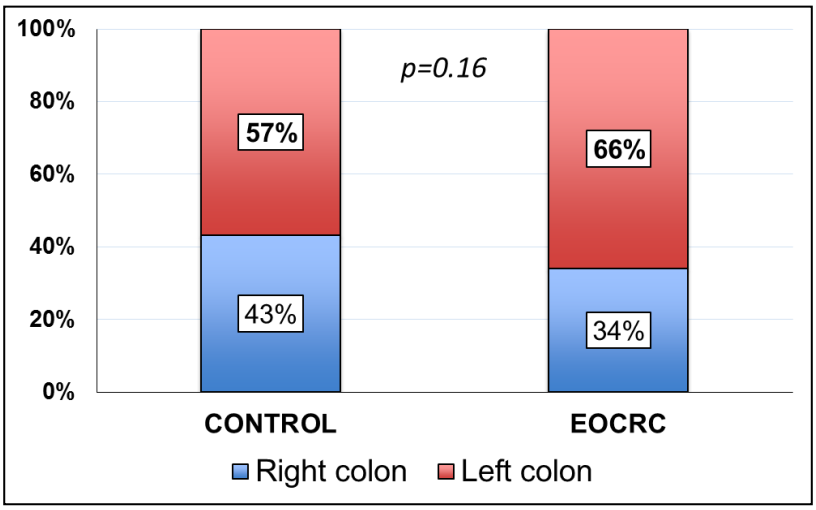
Figure 5. *Gender (B) and age (B) distribution in the two cohorts*



Regarding cancer location, 57% of the control CRCs were located in the left colon, 43% in the right colon and the rectum. The incidence of rectal cancer was not reported separately. When compared to the EOCRC group the difference regarding the tumour location was not significant (p=0.16) (Figure 6). This result is in line with the data currently available from the literature. Indeed, a higher incidence of left colon cancers is present if the cancers related to hereditary syndromes are excluded. In particular, a higher incidence of rectal cancers among individuals under 50 years of age has been reported in several recent studies, reporting also an increase of the incidence in the last decades [41,43,44]. Moreover, higher rates of the right sided CRCs in patients less than 50 years of age are reported in studies that do not exclude tumours associated with Lynch syndrome. In fact,

this syndrome is well known to be associated with a higher incidence of cancers in the right colon in subjects younger than the age of 50, thus, endoscopic screening programs are started at an earlier age. In this study, having excluded cases of CRC-related syndromes, the prevalence of right colon location was not significantly associated with any of the two cohorts.

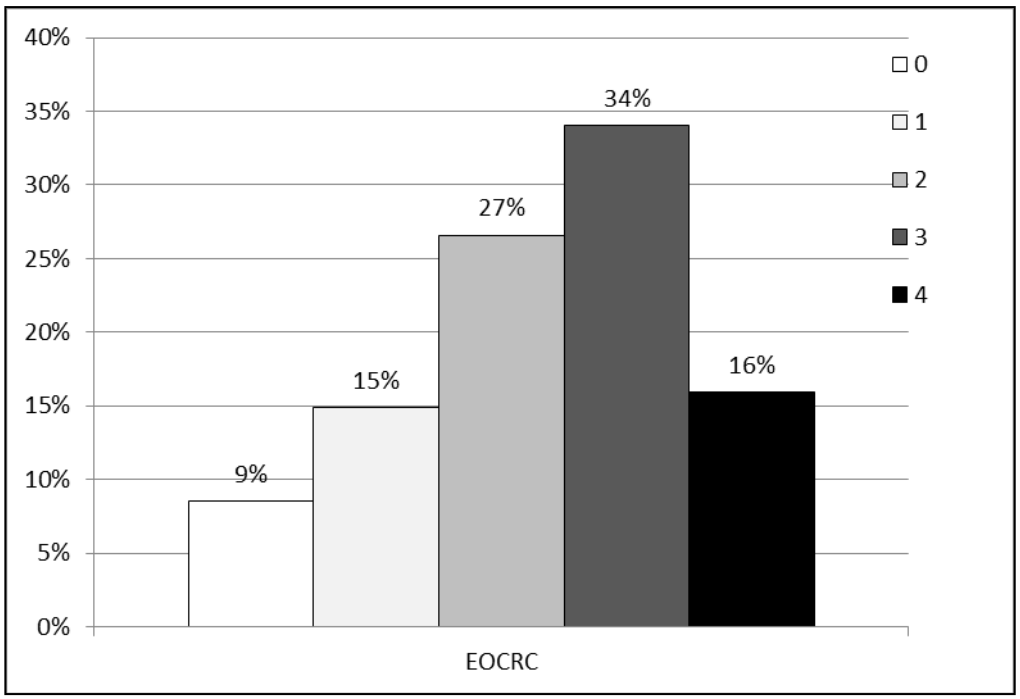
Figure 6. *CRC location in the two cohorts*



As far as tumour stage is concerned, stage II lesions were the most frequent in the control CRC population, accounting for almost half (47%) of the cases, no stage 0 or stage I cancers were present, whereas the advanced stages III and IV were present in 40% and 13% of the cases, respectively. Similarly, in the EOCRCs group stage III and IV lesions represented 50% of patients, although stage IV cancers seem to occur more often (16%) (Figure 7), but this correlation is not statistically significant when compared to the control group (Figure 8). This is in line with the data regarding tumour differentiation, since EOCRCs showed a tendency of higher incidence of poorly differentiated tumours

compared to control CRCs (20% vs. 15%). Nevertheless, this latter difference was not significant (p=0.29) (Figure 9). Finally, in the control group mucinous histology was present in 15% of cases, whereas no singlet cell tumour was found in this group, compared to 21% of mucinous cases and 2 singlet cell cases in the EOCRC cohort.

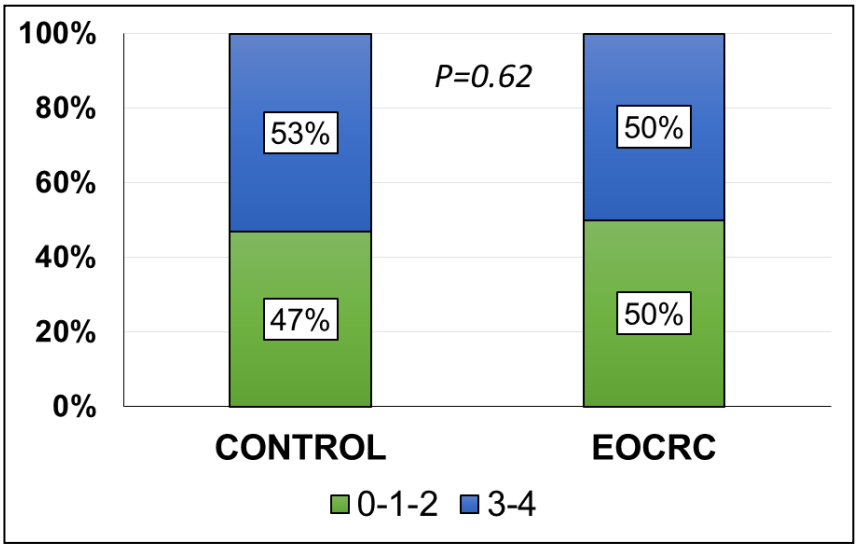
Figure 7. *Tumour stage in the EOCRC group*



This analysis proves that no significant difference occurred between the two groups, although a tendency towards patients with EOCRC presenting with more locally advanced (more stage IV and poorly differentiated tumours) and left sided cancers at the time of diagnosis is detected. More aggressive EOCRC features are found in most population-based studies from Europe, Oceania, Asia, and the United States [41,45-47]. None of the cases in the two groups was diagnosed within a screening program, but only after

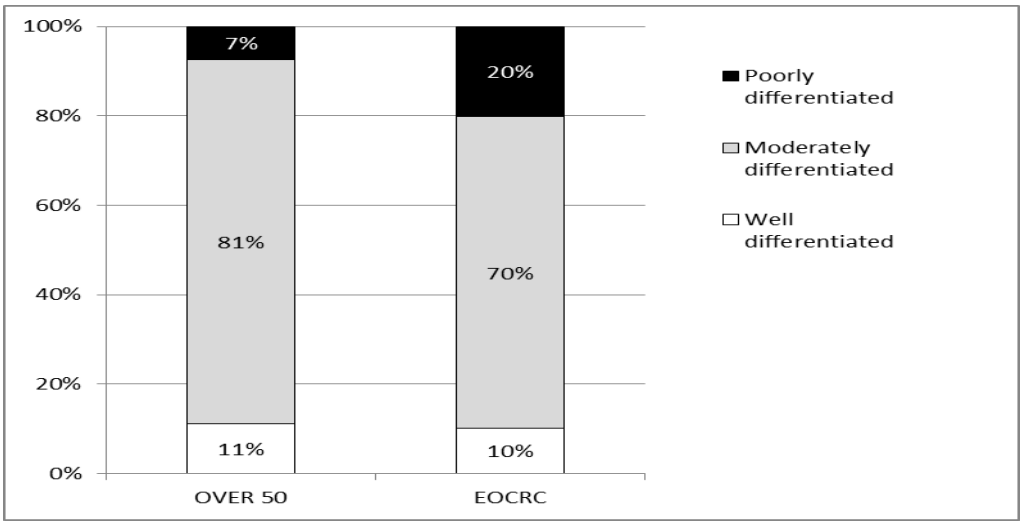
symptoms had developed, which explains the low percentage of cancers at an earlier stage.

Figure 8. *TNM staging in the two cohorts*



It is important to note that nowadays, with the implementation of screening programs, the early CRC detection in the adult population over 50 years of age is rising, whereas the diagnosis of EOCRC cannot be achieved during earlier stages since the screening programs do not include people under 50 unless they have high risk factors such as IBD or strong family history of CRCs. Moreover, the diagnosis of EOCRCs is also delayed due to patient-related factors, such as lack of access to medical check-ups, ignoring symptoms and patient denial, as well as to physician-related factors such as misdiagnosis, that taken together can account for at least 50% of delay in diagnosis [32,41].

Figure 9. *CRC grading in the two cohorts*



4.3 Evaluation of risk factors contributing to EOCRC development

4.3.1 Family history for cancer

A familial history for cancer overall and more specifically for CRC was detected in sporadic EOCRC patients after the careful evaluation performed in this study, despite a lack of reported hereditary conditions at the time of diagnosis. In particular, the EOCRC cohort comprised 17 patients (22%) with a first- or second-degree relative with colorectal cancer outside of a defined syndrome, such as FAP or HNPCC, and 43% with any type of cancer, regardless of location.

Since the data on familiarity was not available for the CRC control group, the comparison was made with data form the regional tumour registry [48], which reports an overall 10% to 15% of familiarity for CRC cases. On the other hand, the data from the literature report that a familial base is present in up to 25% of overall CRC cases [49]. In comparison, the

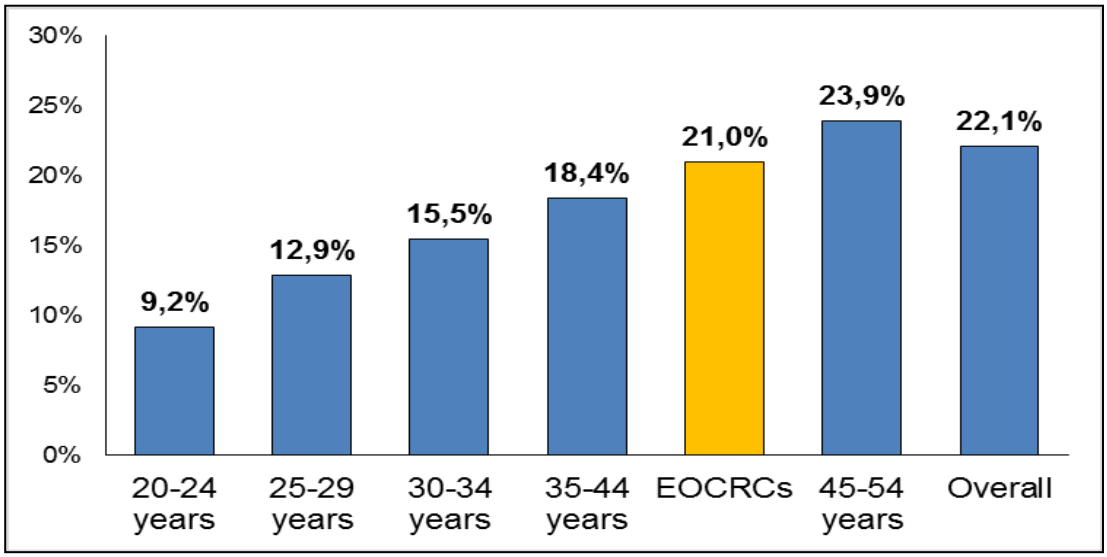
present EOCRC data lies in between the two values, which still confirms that more attention should be made at the time of diagnosis to the presence of a familial risk, also in terms of the genetic counselling of the proband.

4.3.2 Smoking and Alcohol

Smoking rate in the EOCRC group was 36%, while no data were available for the control group. Smoking is a known risk factor for CRC. The incidence of CRC is significantly higher in current as well as former smokers compared with lifelong non-smokers [50]. The prevalence of smokers in the EOCRC group showed to be higher than the overall prevalence of smokers in Italy (20.8%) as reported by the latest data from 2012 of the Ministry of Health [51], and in particular, it was higher for the group of 25-44 years of age that showed 33.7% in males and 24.1% female. Thus, it could be speculated that compared to the average of the national population, patients with EOCRCs are more often smokers and this could be a risk factor that could be worth to further investigate.

Regarding alcohol consumption, in the EOCRC group 21% of patients consumed a minimum of 35 gr of alcohol daily. No data about alcohol consumption was available for the control group.

Figure 10. *Alcohol consumption in the general population [53] and in EOCRCs*



There is a strong scientific consensus acknowledging the association between alcohol drinking and several types of cancer. Similarly, alcohol consumption is associated with a modestly increased risk for development of cancers of the colon and rectum. Regular drinking increases the risk of developing colorectal cancer for 1.5 times as compared to non-drinkers or occasional drinkers [52].

Nevertheless, in this study, alcohol drinking in the EOCRC group appears to be similar to the prevalence in the general population. In particular, daily alcohol consumption increases in the community from 9.2% in the 20-24 years of age group to 23.9% in the 45-54 years of age group, whereas the overall prevalence is 22.1% (Figure 10) [53]. Thus, alcohol consumption does not seem to be a risk factor for development of EOCRC, when compared to healthy population.

4.3.3 Obesity and dyslipidaemias

Epidemiological studies clearly indicate a correlation between obesity and colorectal cancer, and several mechanisms have been proposed and investigated that are linked with obesity-associated insulin resistance and chronic inflammation [10].

In the EOCRC group described in this study, the majority of patients showed a normal weight, with a mean BMI value of 23.4. On the other hand, in the general Italian population the rate of obese and overweight subject is higher among the 35-49 year olds, 9.5% and 31.7%, respectively, as reported by the latest data from the Ministry of Health [54]. Thus, in this study, obesity seems not to be associated with the development of CRC in younger patients.

This result further confirms that the mechanism involved in the association between obesity and colorectal cancers have probably a later effect in life, affecting mainly people over 50 years of age. Nevertheless, it should be noted that this association may be due to different life-styles of younger and older population, and thus may have no direct connection to CRC development. Moreover, the lower weight of subjects with EOCRCs may be due to the neoplastic-related loss of weight and cachexia, due to the later stages of the disease.

4.4 Molecular features

Colorectal cancer related to microsatellite instability is due either to a germline mismatch repair gene mutations, or to 'sporadic' somatic tumour MLH1 promoter methylation. Thus, IHC analysis of the MMR genes is used to detect cancers with MSI. In the two cohorts,

7.2% of cases with lack of MLH1 expression were found in the EOCRC group and 9.9% in the control CRC group. It is interesting to note that all cases of MLH1 deficiency also showed BRAF V600E mutation, thus correlating it to MLH1 promoter methylation [55]. No cases showing lack of expression of MSH2 protein were found in the EOCRC cohort, while 4.7% were present in the control.

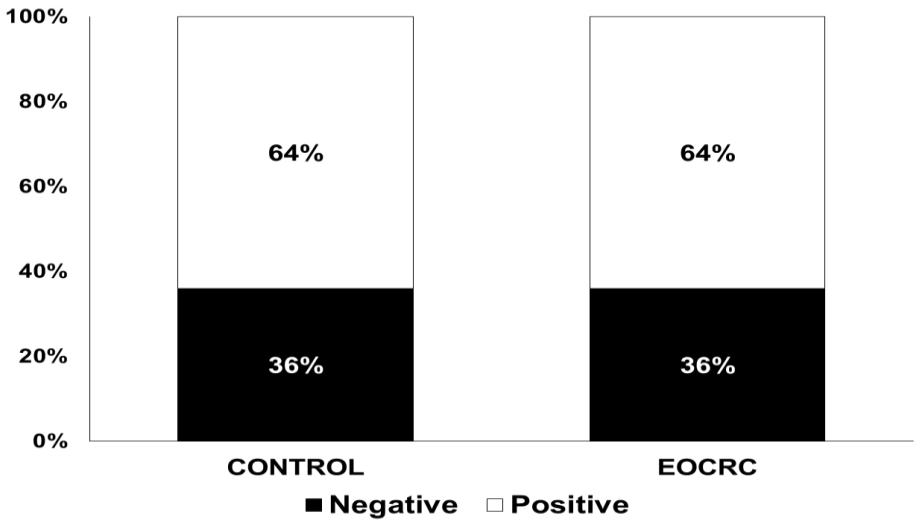
The low number of cases showing lack of MMR proteins reported in this study compared to cohorts described in literature in patients younger than 50 year of age is mainly due to the selection criteria. Indeed, patients known to be part of families with Lynch syndrome were excluded from the analyses since the aim of the project was to characterize specifically the sporadic EOCRCs. Surprisingly, in the control group the number of cases was superior, although overall not significantly different when compared to the EOCRCs ($p=0.22$). An explanation could be that since familial predisposition and the presence of hereditary syndromes is more often investigated for CRCs developing in younger subjects than in patients older than 50 years, thus the control group probably comprised a few unknown hereditary tumours.

KRAS mutation is an early event in the development of colorectal cancer and literature reports 30% to 40% of positive CRC cases. However, the prognostic role for KRAS in CRC remains controversial. On one hand, mutations in KRAS have been associated with a more advanced disease and a worst outcome. On the contrary recent studies showed that *KRAS* mutation was not a prognostic factor for therapy outcome [43,56,57].

The data on KRAS analyses were available for 48 cases of the EOCRC and showed *KRAS* mutation in 18 cases (37%). This result is in line with the literature reporting

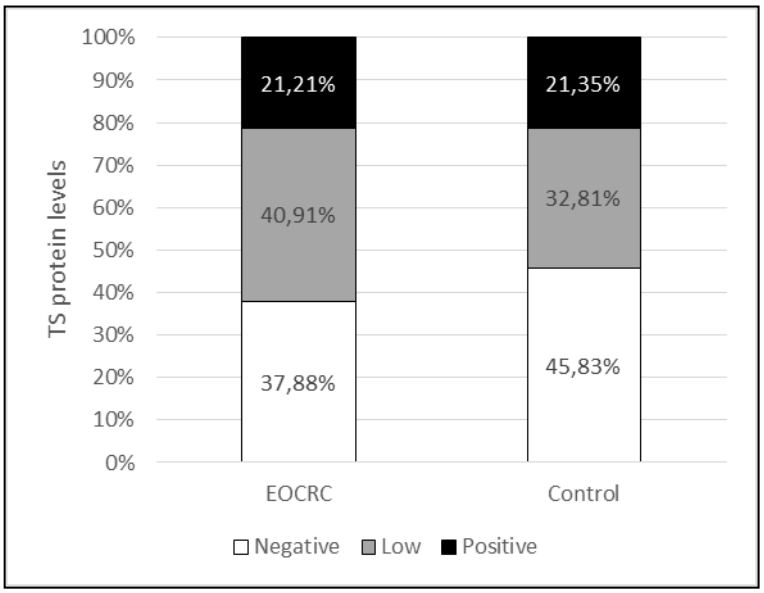
association of KRAS with more aggressive cancers, since EOCRC cohort showed a tendency of a high incidence of advanced disease.

Figure 12. *p53 protein levels in the two cohorts*



No substantial differences were found between EOCRC and control CRC groups regarding the IHC levels of the proteins routinely requested by the oncologist to guide treatment, namely p53 and TS. Literature reports alterations of p53 protein levels to be associated with worse survival for CRC patients treated with chemotherapy [58]. In both groups analysed in this study, IHC analysis showed low p53 levels in 36% of cases (Figure 12). This indicates that the loss of p53 is not correlated to the age of onset of CRC. Thymidylate synthase (TS) is the target of the active metabolite of 5-fluorouracil, and its level of expression is the most important determinant of clinical efficacy of this drug. Low levels of TS in the tumour of patients with CRC is a predictor of response to treatment with 5-FU, while high levels of TS in tumour correlate with a poor response to treatment [39].

Figure 13. *Thymidylate synthase (TS) protein levels in the two cohorts*



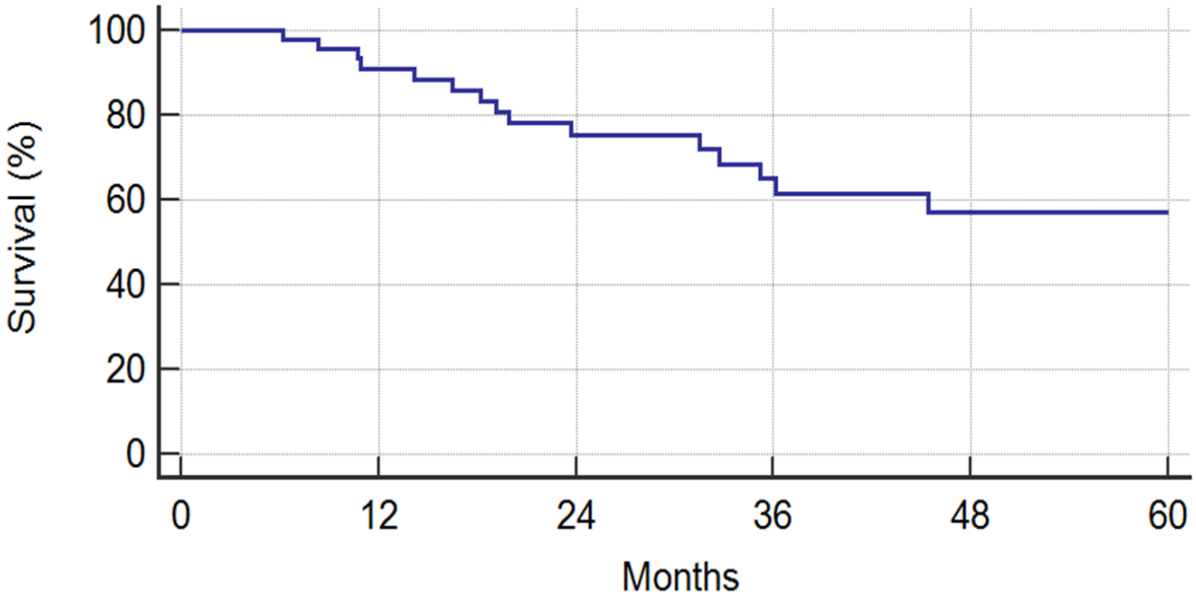
In this study population, the TS levels were similar between the two groups (Figure 13); therefore, it is unlikely that this marker could affect the response to treatment in the two groups. However, the role and the effect of these markers among the two groups needs still to be clarified.

4.5 Follow-up and oncologic outcome

After surgical intervention, EOCRC patients were transferred to the Oncological department either to undergo chemotherapy or simply to continue follow-up. Patients were followed to their date of death or their last contact with the medical centre. The median follow-up was 35 months (range 4 to 110). The relatively short follow-up of EOCRC patients could be due to the presence of a more advanced disease and thus the survival is

expected to be shorter. Secondly, younger patients who feel well after surgery are usually less compliant to completing the therapy due to side effects.

Figure 14. *Overall survival of EOCRCs*

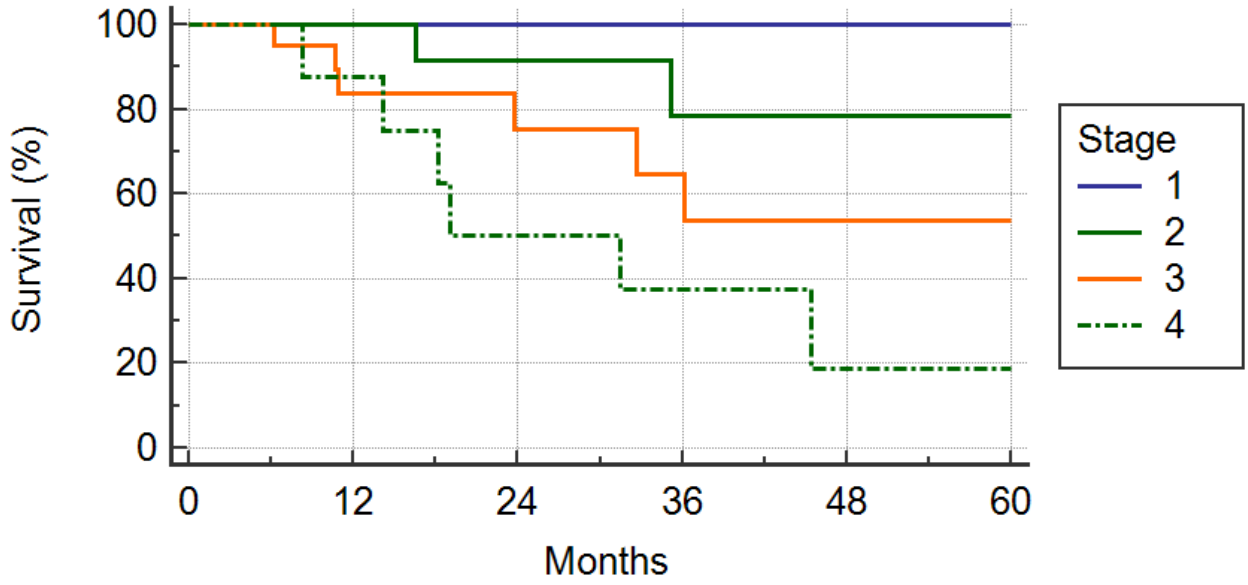


Adjuvant chemotherapy was undertaken by 49% of patients in the EOCRC groups.

The overall the 1-year survival was 91%, while at 5 years it dropped to 57% (Figure 14). The survival rate divided by tumour stage showed a survival rate of 100%, 79%, 54% and 19% for stages I, II, III and IV, respectively (Figure 15).

No significant survival rate differences were observed when comparing p53 levels, TS levels, MMR expression or cancer location.

Figure 15. *Stage survival of EOCRCs*



The overall survival of the EOCRCs appears to be lower when compared to the overall survival reported for colorectal cancer in Italy, where the 5-year survival is 64% in men and 63% in women according to the latest data [59]. These latter data include also cases diagnosed within the screening program, therefore diagnosed at earlier stage which is one parameter explaining the better survival. In fact, the overall survival before the introduction of the screening programs was around 58%, closer to the EOCRC rate reported in this study.

5 Concluding remarks

The increasing occurrence of CRC developing in young patients with no identified genetic predisposition demands maintaining a high index of suspicion when people below 50 years of age present with symptoms. Indeed, EOCRCs appear frequently as an aggressive disease located in the sigmoid colon and rectum, and most patients are symptomatic at the time of presentation, mainly presenting with rectal bleeding, haematochezia or abdominal pain.

The genetic basis in the majority of early onset colorectal carcinomas remains unknown, however, most EOCRCs, not related hereditary syndromes, appear to arise through the same pathways as sporadic CRCs, such as the classical adenoma-carcinoma sequence, but with only rare involvement of the methylator pathway.

Taken together, the analyses described in this study suggest that, in the absence of screening programs for patients under 50 years of age, the risk factor of a family history and the presence of symptoms may be considered as an indication for prompt endoscopic investigation in these patients, since this may reduce the stage of disease at diagnosis and likely have an impact on improving survival.

6 References

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