UNIVERSITY OF PISA

FACULTY OF MEDICINE AND SURGERY

PhD school in:

“FISIOPATOLOGIA CLINICA E SCIENZE DEL FARMACO”

School Dean: Prof. P. Miccoli

Program:

“SCIENZE CHIRURGICHE, ANESTESIOLOGICHE E DELL’EMERGENZA”

Title:
Epigenetic biomarkers, folates and genetic susceptibility in colorectal carcinoma

Supervisors:

Prof. Lucia Migliore

Prof. Paolo Miccoli

Candidate:

Francesca Migheli

Academic Year 2011 - 2012

Index

Abstract I

Introduction 1

1. Colorectal cancer 1

1.1 Epidemiology 2

1.2 Genetics of colorectal cancer 4

1.3 Cytogenetics of colorectal cancer 10

1.4 Epigenetics 11

1.4.1 DNA methylation in colorectal cancer 13

1.4.2 Histone modifications in colorectal cancer 18

1.4.3 NcRNA alterations in colorectal cancer 20

1.5 Colorectal adenoma-carcinoma sequence 21

1.5.1 *APC* (Adenomatous Polyposis Coli) gene 23

1.5.2 *MGMT* (O-6-methylguanine-DNA methyltransferase) gene 25

1.5.3 *CDKN2A* or $p16^{Ink4A}$ (Cyclin-dependent kinase inhibitor 2A) gene 28

1.5.4 *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1) gene 30

1.5.5 *hMLH1* (mutL homolog 1, colon cancer, nonpolyposis type 2) gene 31

1.6 Environmental factors that might influence epigenetic patterns in colorectal cancer 33

1.7 Genetic factors affecting DNA methylation in colorectal cancer 43

2. DNA methylation analysis techniques 45

Materials and Methods 48

1. Study population 48

2. Isolation of human epithelial cells 50

3. Extraction of genomic DNA 51

4. Genotyping 51

5. Methylation analysis 52

5.1 MS-HRM protocols 53

5.2 Pyrosequencing protocols 59

6. Statistical analysis 66

Aim of the study 67

Results 69

1. Comparison between tumor and healthy tissue in CRC patients 69

2. Correlation between methylation and clinical-pathological features 72

3. Comparison between MS-HRM and Pyrosequencing techniques 76

4. Tumor cells separation 79

5. Polymorphisms in folate metabolism genes and methylation 80

6. Folate, homocysteine and vitamin B12 values in CRC patients 86

Discussion 88

References 96

Abstract

The implications of DNA hypomethylation and hypermethylation in the etiology of tumorigenesis have become quite clear. Since epigenetic modifications are reversible, methylation studies are extremely promising to better characterize colorectal cancer (CRC) and to identify new tools for diagnosis and prognosis. In the frame of a wider study ongoing aimed at searching for possible correlations among genetic, epigenetic and environmental factors in colorectal cancer, this thesis took into account a cohort of CRC subjects until now recruited, focusing on the following items: 1) the validation of the MS-HRM protocol, used for the methylation analysis, by comparing it with a widely employed technique (Pyrosequencing); 2) the evaluation of the influence of an immunomagnetic method with microbeads coated with the antibody CD326+, specific for epithelial cells, to clarify if cancer epithelial cells from the surgically resected CRC tissue could give more accurate results in DNA methylation levels detection with respect to the whole CRC tissue; 3) the detection of methylation levels (performed by MS-HRM) in promoters of of *APC*, *CDKN2A, hMLH1, MGMT* and *RASSF1*A genes in CRC and healthy adjacent tissue specimens; 4) the analysis of the correlation among the methylation status of the chosen genes and the clinical-pathological features of the patients; 5) the analysis of the correlation among *MTHFR* C677T, *DNMT3B* C-149T polymorphisms and the methylation levels of *APC, CDKN2A, hMLH1* and *MGMT* gene promoters. Results obtained by using MS-HRM and Pyrosequencing have shown to be quantitatively comparable. No statistically significant difference between the epithelial cells CD326+ fraction and the whole tissue about the promoter methylation of *APC, CDKN2A, MGMT* and *hMLH1* genes was observed, although some patients showed individually a statistically significant difference between the two experimental

conditions regarding the degree of methylation of these genes. It was also seen, for all of the studied genes, a higher methylation level in CRC tissue respect to the healthy adjacent tissue. No statistically significant association between stage (TNM), gender, sex, tumor size, and location with regard to the methylation profile of each of the analyzed genes was found in the examined cohort. However, it was found an interesting positive association between age and both *hMLH1* (*P value*= 0.007) and *MGMT (P value= 0.03).* Finally significant interactions between *MTHFR* 677C>T, DNMT3B -149C>T polymorphisms and gene promoter methylation were observed.

Introduction

1. Colorectal cancer

The digestive surface of the human large intestine is characterized by a monolayer of specialized epithelial cells that forms crypts. The first recognizable manifestation of epithelial alteration during colorectal tumor development are the Aberrant Crypt Foci (ACF), small hyper- or dysplastic lesions. Larger ACF with altered morphology, dysplastic histology and associated gene mutations are high-risk candidates for adenoma and CRC formation. According to the their architecture, adenomas may be divided in tubular, when coarsely lobulated and pedunculated, or villous, when sessile, covering a broad area directly onto the muscularis mucosae (the muscle layer underlying the epithelial lining) and submucosa (the underlying stromal layer). Villous adenomas are thought to have higher risk of malignant progression. The carcinoma in situ are advanced high dysplastic lesions still confined within the epithelial layer. Finally, malignant adeno-carcinomas are characterized by the ability to invade the surrounding tissues through the muscularis mucosae and into the stromal compartment, and migrate to distal organs where they can form metastasis. The TNM Classification of Malignant Tumours (TNM) is a cancer staging system that describes the extent of cancer in a patient's body:

- T describes the size of the tumor and whether it has invaded nearby tissue,
- N describes regional lymph nodes that are involved,
- M describes distant metastasis (spread of cancer from one body part to another).

Particularly T represents the size or direct extent of the primary tumor (Tx: tumor cannot be evaluated, Tis: carcinoma in situ, T0: no signs of tumor and T1, T2, T3, T4: size and/or extension of the primary tumor); N represents the degree of spread to regional lymph nodes (Nx: lymph nodes cannot be evaluated, N0: tumor cells absent from regional lymph nodes, N1: regional lymph node metastasis present, N2: tumor spread to an extent between N1 and N3, N3: tumor spread to more distant or numerous regional lymph nodes; M represents the presence of metastasis (Mx: distant metastasis cannot be evaluated, M0: no distant metastasis, M1: metastasis to distant organs (beyond regional lymph nodes).

The Stage grouping is as follow:

Stage 0: Tis N0 M0

Stage I: T1 N0 M0; T2 N0 M0: Cancer has begun to spread, but is still in the inner lining.

Stage II: T3 N0 M0; T4 N0 M0: Cancer has spread to other organs near the colon or rectum. It has not reached lymph nodes.

Stage III: any T, N1-2, M0: Cancer has spread to lymph nodes, but has not been carried to distant parts of the body.

Stage IV: any T, any N, M1: Cancer has been carried through the lymph system to distant parts of the body. This is known as metastasis. The most likely organs to experience metastasis from colorectal cancer are the lungs and liver.

1.1 Epidemiology

CRC is the third most common cancer in men (663 000 cases, 10.0% of the total) and the second in women (571 000 cases, 9.4% of the total) worldwide (data observed in 2008). Almost 60% of the cases occur in developed regions. The highest percentage is observed in Australia/New Zealand and Western Europe, the lowest in Africa (except Southern Africa) and South-Central Asia. Colorectal cancer is the fourth most common

cause of death from cancer (608 000 deaths worldwide, the 8% of all cancer deaths). Mortality rates are also lower in women than in men, except in the Caribbean. There is less variability in mortality rates worldwide (6-fold in men, 5-fold in women), with the highest mortality rates in both sexes estimated in Central and Eastern Europe and the lowest in Middle Africa (Figure 1). Table 1 shows the incidence, mortality and prevalence in the world for the year 2008 (Cancer Mondial, Globocan 2008).

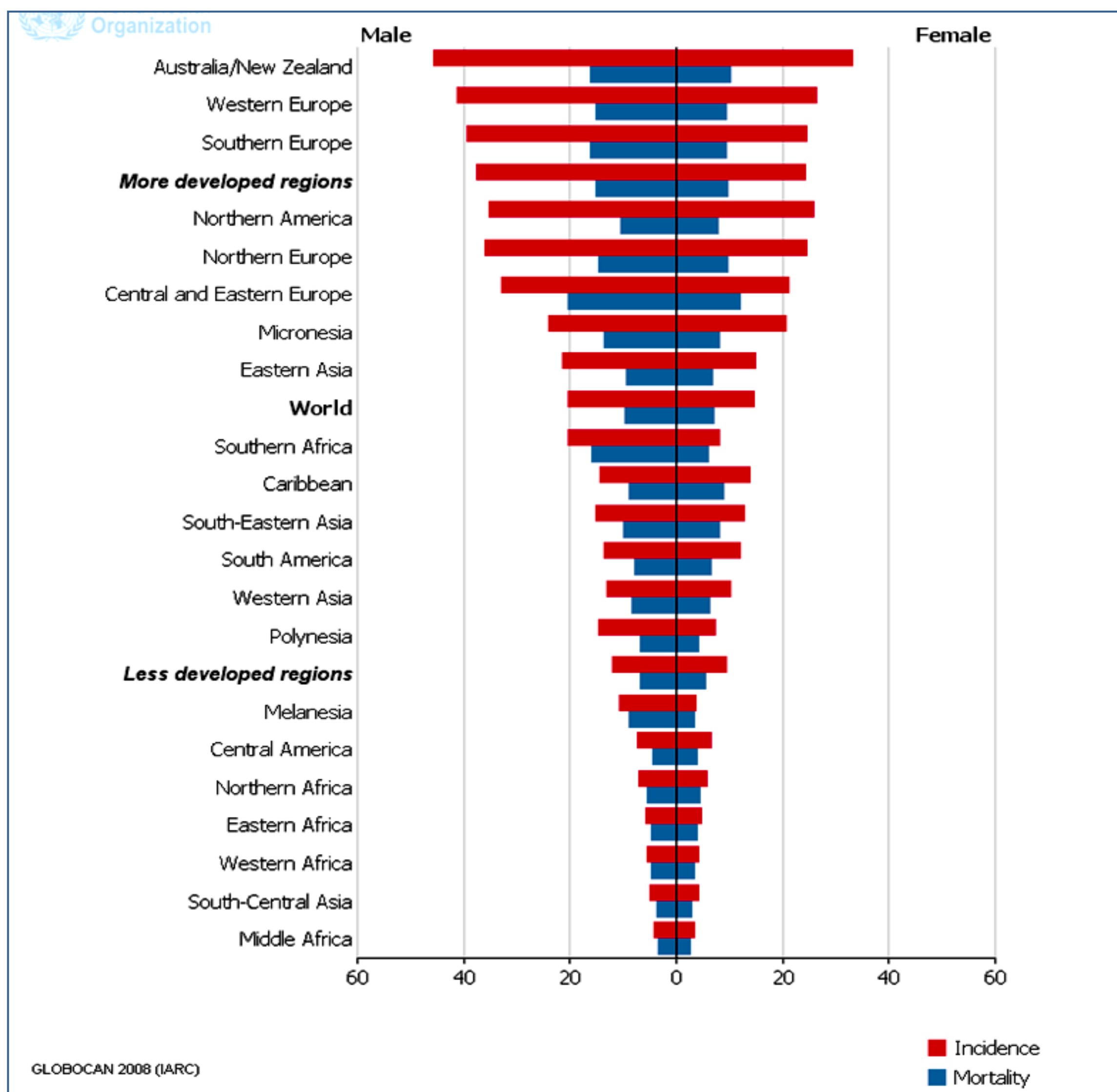


Figure 1: Estimated age-standardised rates (World) per 100,000

Table 1: Colorectal Cancer Incidence, Mortality and Prevalence Worldwide in 2008.

Estimated numbers	**Men**			**Women**			**Both sexes**		
	Cases	**Deaths**	**5-year prev.**	**Cases**	**Deaths**	**5-year prev.**	**Cases**	**Deaths**	**5-year prev.**
World	663	320	1765	571	288	1495	1234	608	3260
More developed regions	389	165	1141	338	154	968	727	319	2109
Less developed regions	274	154	624	232	134	526	506	288	1150
WHO Africa region	14	11	30	12	9	24	26	20	54
WHO Americas region	122	46	360	118	49	342	240	95	702
WHO East Mediterranean region	13	9	29	10	7	23	23	16	52
WHO Europe region	238	115	645	212	107	564	450	222	1209
WHO South-East Asia region	50	34	92	47	32	89	97	66	181
WHO Western Pacific region	224	101	607	170	81	450	394	182	1057
IARC membership (22 countries)	372	154	1082	319	143	909	691	297	1991
United States of America	79	24	245	74	26	227	153	50	472
China	125	61	289	95	48	219	220	109	508
India	20	14	27	16	11	21	36	25	48
European Union	182	80	507	151	68	417	333	148	924

1.2 Genetics of colorectal cancer

Most cases of colorectal cancer are sporadic. Risk factors include increasing age, male sex, previous colonic polyps or previous colorectal cancer, and environmental factors (red meat, high-fat diet, inadequate intake of fiber, obesity, sedentary lifestyle, diabetes mellitus, smoking, and high consumption of alcohol). Inflammatory bowel disease (ulcerative colitis and Chron's disease) are two-thirds of the incidence, and the risk increases with duration of illness, severity and extent of inflammation.

A number of familial syndromes are associated with a high risk of colorectal adenocarcinoma. FAP (Familial Adenomatous Polyposis) is an autosomal dominant condition due to different types of mutations in *APC* gene (Adenomatous polyposis

coli), which encodes a tumor suppressor that is part of the WNT signaling pathway (see below); its incidence varies from 1 in 7000 to 1 in 22000 births. The syndrome is more common in Western countries and it is characterized by more than 100 adenomatous polyps in the colon and rectum, developing after 10 years age. In addition to colonic polyps the syndrome can give rise to extraintestinal manifestations such as osteomas, epidermoid cysts, supernumerary teeth, thyroid tumors and brain tumors. By age 10 years, 15% of FAP gene carriers manifest adenomas; by age 20 years, the probability rises to 75% and by 45 years the risk becomes 90% (www.cancer.gov). The average age of CRC diagnosis if untreated is 39 years; 7% develop CRC by age 21 and 95% by age 50 (Jasperson *et al.*, 2010). The clinical features of FAP appear to be generally associated with the location of the mutation in the *APC* gene and the type of mutation; for example a dense carpeting of colonic polyps is seen in patients with mutations between codons 169 and 1393. Figure 2 shows mutations in different regions of *APC* gene that correspond to variation in severity of symptoms.

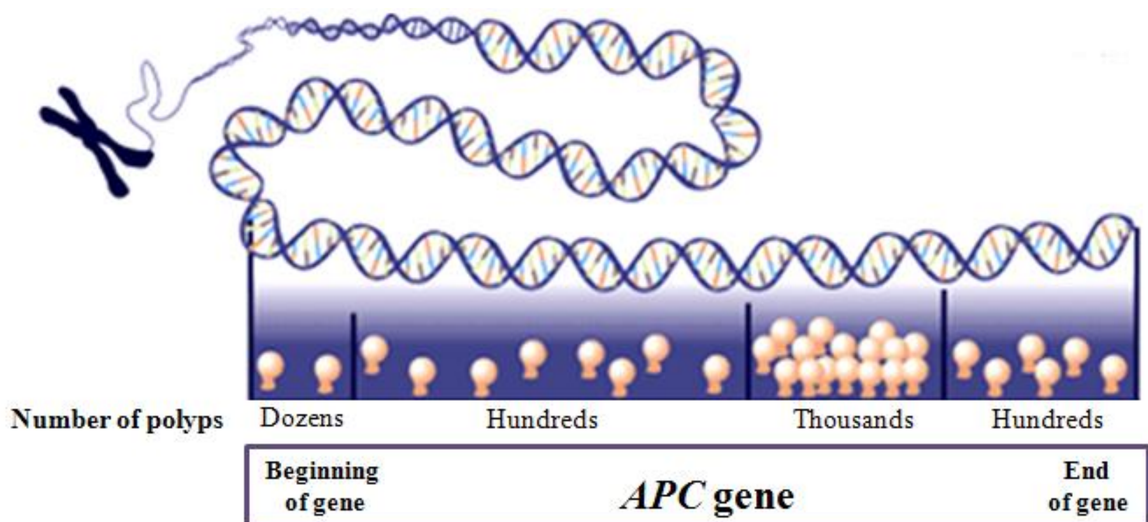


Figure 2: Mutations in *APC* gene cause FAP, but mutations in different regions of the gene will have a varying effect on the nature and severity of symptoms.

The detection of *APC* mutation is approximately 80% using sequencing alone, such as the protein truncation assay (PTT). Studies reported whole exon deletion in 12% of

patients negative to *APC* sequencing test. Individuals at risk of FAP begin surveillance in the early teenage years (10-15 years). Colon surveillance should not be stopped in carriers of *APC* mutations because polyps sometime do not manifest until the fourth and fifth decades of life. Colectomy could be important when, in a FAP patient, numerous polyps have developed (>20) and when adenomas >1 cm are found or when advanced histology appears. Cyclooxigenase II inhibitors such as celecoxib and rofecoxib have been associated with a decrease in polyp size and number in FAP patients. Attenuated FAP is characterized by fewer adenomatous polyps (30, range 0-100) at colon and rectum than in classic FAP and is associated with particular subsets of *APC* mutations, including missense changes. Approximately 7% to 17% of patients with a FAP phenotype and without a detectable *APC* germline mutation carry the mutations in *MYH* gene.

MYH associated polyposis (MAP) occurs in about 1/18000 CRC patients. MAP is caused by biallelic mutations in *MYH*, a gene involved in the base excision repair pathway to defend DNA against oxidative damage. This gene product prevents G:C to T:A transversions, caused by oxidative stress. The colonic phenotype of MAP mimics attenuated FAP, including propensity for proximal colonic neoplasms. There is also an association between hyperplastic or sessile serrated polyps (Jasperson *et al.*, 2010).

Individuals with Lynch syndrome (hereditary non-polyposis colorectal cancer/HNPCC) are predisposed to various types of cancers and are the 2-4% of all CRCs; in particular this syndrome occurs in about 1/300 people with colorectal cancer. Endometrial cancer is the most common extracolonic malignancy associated with Lynch syndrome; also other cancers such as gastric, ovarian, biliary, urinary tract, small bowel, brain are associated with HNPCC. It is an autosomal dominant condition caused by mutations in one of several DNA mismatch repair (MMR) genes. The mean age of CRC diagnosis in

HNPCC syndrome mutation carriers is 44 years, compared with 64 years in sporadic cancer. Lifetime CRC risk is estimated to be 50-80%. Colon cancers arise in Lynch syndrome at a younger age of onset and a more proximal location compared to sporadic neoplasms; these types of tumor are poorly differentiated, mucinous. They are also characterized by a high level of microsatellite instability (MSI-H), a feature of cancers with mutations in MMR, necessary for maintaining genomic fidelity by correcting single base mismatches and insertion-deletion loops that form during DNA replication. Mutations in h*MLH1* and hMLH2 account for the up to 90%. Mutations in hMSH6 account for approximately 10% and mutations in hPMS2 are detected in rare occasions. The research criteria for defining Lynch syndrome families were established by the international collaborative group (ICG) meeting in Amsterdam in 1990 and are known as the Amsterdam criteria.

Amsterdam criteria:

1) One member diagnosed with colorectal cancer before age 50 years.
2) Two affected generations
3) Three affected relatives, one of them a first degree relative of the other two
4) FAP should be excluded
5) Tumor should be verified by pathological examination

To increase sensitivity, the Amsterdam criteria were revised to obtain Amsterdam criteria II:

1) There should be at least three relatives with a Lynch syndrome associated cancer (colorectal cancer or cancer of the endometrium, small bowel, ureter, or renal pelvis).
2) One should be a first-degree relative of the other two
3) At least two successive generations should be affected

4) At least one should be diagnosed before age 50 years
5) Familial adenomatous polyposis should be excluded in the colorectal cancer cases
6) Tumor should be verified by pathological examination.

Another set of clinical criteria (Bethesda Guidelines) can be used to identify families with germline MMR mutations:

1) Colorectal cancer diagnosed in an individual younger than 50 years.
2) Presence of synchronous, metachronous colorectal, or other Lynch syndrome-associated tumor (i.e endometrial, stomach, etc) in an individual regardless of age.
3) Colorectal cancer with MSI-high pathologic associated features diagnosed in an individual younger than 60 years
4) Colorectal cancer or Lynch syndrome associated tumor diagnosed in at least one first-degree relative younger than 50 years.
5) Colorectal cancer or Lynch syndrome-associated tumor diagnosed at any in two first degree, second-degree relatives.

Genetic tests typically start with analysis of *hMLH1* and *hMLH2* by sequencing. Another approach for identifying Lynch syndrome, is to perform tumor testing when any of the Bethesda guidelines are identified. Approximately 90% Lynch syndrome associated CRC will have MSI-H, making this analysis very sensitive; sporadic MSI-H CRCs could be the result of somatic hypermethylation of the *hMLH1* promoter region. Tumor testing with IHC utilizes four antibodies specific for hMLH1, hMSH2, hMSH6, hPMS2 proteins to evaluate tumor for MMR deficiency. Surveillance decrease both incidence and related deaths. Colonoscopies at 3-years intervals have been shown to decrease the risk of CRC by 50% and prevent CRC deaths. The reduction in CRC risk

and death is expected to be even greater when screening intervals are reduced to every 1-2 years. Screening colonoscopy in affected individuals should be initiated by 20-25 years of age and repeated every 1-2 years. Subtotal colectomy with ileorectal anastomosis is advised with the appearance of colon cancer. Population-based studies have led to estimates that 0.8%-2.3% of all CRC cases meet the Amsterdam criteria I and II for Lynch syndrome. A subset of these (40-70%) do not have MMR deficiency and have been termed familial colorectal cancer type X (that have lower CRC risk than HNPCC and do not exhibit MSI). Familial CRC arises from a number of different, lower-penetrance susceptibility genes than those associated with the well-defined, but rare inherited syndromes. No specific genetic markers are available for common familial CRC, then screening and surveillance are based on family history. Patients with a single first-degree relative older than age 60 years with colon cancer should receive average-risk colon cancer screening, but starting at age of 40 year; patients who have one relative with CRC before 60 years or two first degree relatives with CRC should be screened every 5 years by colonoscopy, starting at age 40 years, or at age 10 years younger than the earliest case in the family; patients with only second or third degree relatives with CRC should receive average-risk screening. The recommendations for screening average risk persons include these options: fecal occult blood screening each year, followed by colonoscopy; double contrast barium enema every 5-10 years; colonoscopy every 10 years.

Rare Colon Cancer Syndromes: Peutz-Jeghers syndrome (PJS) is an early-onset autosomal dominant disorder characterized by small bowel and histologically distinctive hamartomatous polyps. Individuals with PJS have an estimated 81-93% lifetime risk of cancer. Germline mutations in the *STK11* gene at chromosome 19p13.3 (tumor suppressor gene) have been identified in approximately half of PJS families; a

study reported that 85% of individuals with PJS developed cancer by age 70. Juvenile polyposis syndrome (JPS) is a genetically heterogeneous, rare, childhood-onset, autosomal dominant disease due to germline mutations in the *MADH4* gene (SMAD4/DPC4) in approximately 15-20% of cases and to mutations in *BMPR1A* gene in approximately 25-40% of cases. HPP is a rare condition characterized by multiple and/or large hyperplastic polyps of the colon. The World Health Organitation's criteria for HPP include 30 cumulative hyperplastic polyps of any size distributed throughout the colon; >5 hyperplastic polyps proximal to the sigmoid colon with at least two >10 mm in diameter; or at least 1 hyperplastic colonic polyp in an individual with a first-degree relative with HPP. These conditions increase risk of CRC, which occurs on average in the 50s or 60s. The inheritance of HPP is weak, although both recessive and dominant transmission patterns have been proposed. Curiously, individuals with biallelic MUTYH mutations have been shown, on occasion, to meet criteria for HPP (Cunningham *et al.*, 2010; Jasperson *et al.*, 2010; www.cancer.gov).

1.3 Cytogenetics of colorectal cancer

For colorectal cancers, the acquisition of genomic instability is considered a key hallmark. Three major molecular subtypes can be recognized: MIN (or MSI, for "microsatellite instability"), CIN (for "chromosomal instability") and CIMP (for "CpG islandmethylator phenotype"). MIN-CRC accounts for approximately 15%–20% of sporadic colorectal cancers. The characteristics of the three pathways (MIN, CIN, and CIMP) are not completely defined and thus they are not mutually exclusive; it is believed that a tumor can occasionally show features of multiple pathways, although the extent and nature of this overlap remains to be determined. CIN is the most common type of genomic instability observed in colon cancer and occurs in 80%–85% of

colorectal tumors. It occurs mainly in non-MIN cancers (or MSS for "microsatellite stable") which are proficient for mismatch repair. CIN CRC show several forms of genomic instability, characterized mainly by chromosomal rearrangements and numerical abnormalities at a greatly increased rate compared with normal cells. The most recurrent aberration found in all cytogenetic studies performed, either in primary tumors or in colon cancer cell lines or in fixed colorectal cancer tissue blocks is 18q (Figure 3). (see the review by Migliore *et al.*, 2011).

Chromosome loss	Chromosome gain
18, 17p, 1p, 4, 14, 5q, 21	7, 12, X, 5, 8
18q21	
	20q13
18q	20q
18p21-pter, 15q11-q21, 17p12-13, 18q12-21	8q23-ter, 13p14-31, 20q13
4, 18p, 14q	17p, 17q, 1q11, 12p, 19
8p, 18q, 1p22, 4q26, 15q21	20, 8q, 8q28, 16q24.3, 20q13
18q	13q
18, 17p, Y, 1p3, 8p	13, 20, 7, X, 12, 6
8p, 18, 18q	3, 3q, 5, 5p, 5q, 7, 8q, 20, 20q, 13, X
4, 5, 8, 10, 14, 15, 17, 18, 21, 22, Y, 18q10 [i(8) (q10)], 17q10 [i (17) (q10)]	7, 13, 20, X

Figure 3: The most frequent aberrations found in CRC (Migliore *et al.*, 2011).

1.4 Epigenetics

Epigenetics is defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. An epigenetic modification is DNA methylation, a covalent addition of a methyl group (CH3) to the nucleotide cytosine. In mammals, most of the DNA CpG sites are methylated (90%–98%), but there are specific CpG-rich areas of DNA where most CpGs are not methylated (CpG islands); a few genes are imprinted genes, regulated by methylation of the CpG islands in their promoter, and the markings are stably replicated during cell division, but are reversed when inherited

through an individual of the opposite sex. CpG islands are associated with promoter regulatory regions of almost all housekeeping genes as well as with half of tissue-specific genes (Esteller *et al.*, 2011). Promoter hypomethylation has been associated with an increased gene transcription. DNA hypermethylation occurs at specific regulatory sites in the promoter regions or repetitive sequences. A heavy density of cytosine methylation in the CpG islands of the tumor suppressor gene promoters can lead to a complete block of transcription, and many types of cancer use this mechanism to inactivate tumor suppressor genes. Another critical epigenetic mechanism refers to chemical modifications of the histone tails. Histones, besides being DNA-packaging proteins, can regulate the underlying DNA sequences through complex posttranslational modifications of their N-terminal tails, such as aminoacid-specific acetylation, methylation, or phosphorylation. Histones are acetylated on lysine residues at their amino termini by histone acetyltransferases (HATs), and acetylated histones are deacetylated by HDACs. The opposing effects of HATs and histone deacetylases (HDACs) regulate gene expression through chromatin modification. The HDAC-mediated removal of acetyl groups from lysine residues in the amino termini of histones leads to chromatin condensation and transcriptional inactivation of the involved DNA (Ropero *et al.*, 2007; Yang *et al.*, 2007). This transcriptional inactivation can contribute to suppression of tumor suppressor gene expression and enhanced tumorigenesis. Non-coding RNA (ncRNAs) are involved in the regulation of many important biological processes; the most widely studied class of ncRNAs are miRNAs, which are involved in post-transcriptional gene silencing by controlling mRNA translation into proteins. Alterations (genetic or epigenetic) of genes coding these ncRNAs can modify the expression profile of the ncRNAs and thus alter the mechanism they regulate.

1.4.1 DNA methylation in colorectal cancer

Almost 30–40% of proximal site colon tumors and a 3–12% of distal colon and rectal tumors are characterized by a high CIMP, in which numerous CpG islands are methylated and several tumor suppressor genes or ncRNA are inactivated. Altered promoter DNA methylation seems to correlate with deregulation of DNA methyltransferases. *De novo* DNA methyltransferase 3B (DNMT3B) is generally repressed in human colorectal cancer cell lines (CCL) and primary tumors by aberrant DNA hypermethylation of its distal promoter. *DNMT3B* distal promoter region was unmethylated in nomal colon tissue and densely hypermethylated in certain colon cancer cell lines. The CpG sites located 764 bp upstream and 208 downstream the TSS (transcription start site) were hyper-and hypomethylated respectively and showed no differences between healthy and tumor samples. The CpG site 352bp upstream of the TSS was completely unmethylated in non–tumorigenic colon tissues, while it was hypermethylated to different degree in most colon CCL analyzed (HCT15, Sw480, Co115, HT29). None of the tumors overexpressing *DNMT3B* showed *DNMT3B* promoter hypermethylation. 25% of primary colon tumors with an unmethylated *DNMT3B* promoter overexpressed *DNMT3B*. At the epigenome level, *DNMT3B* promoter hypermethylation was associated with the hypomethylation of gene promoters usually hypermethylated in the healthy colon. Forced *DNMT3B* overexpression in cancer cells restored the methylation levels of these promoters in the healthy colon (Huidobro *et al.*, 2012). Some genes such as *hLMH1*, *MGMT* and *TSP1* showed an increase in methylation during all the stages of the disease. Other genes such as *RASSF1A* or *TIMP3* seem more methylated in the last stages or in metastases. However, sometimes conflicting results are found, with the same gene studied. This could be because of the analysis of different CpG sites in the same gene depending on the

different methods used to assess methylation [Pyrosequencing, vs Methylation Specific Polymerase chain reaction or vs COBRA]. Moreover, differences in age, tumor type or heterogeneity, or different exposure to environmental factors (diet or microorganisms) could also explain the contrasting methylation pattern of specific genes found in various studies. Leong and co-workers (Leong *et al.*, 2011) found an inverse relationship between methylation of 10 tumor suppressor genes and chromosomal aberrations; then if the frequency of methylation is less prevalent in advanced disease, it is possible that the advanced rectal cancers are driven via the CIN pathway, whereas the early cancers were driven via the methylation pathway. Some genes could have a dual role to promote or suppress tumor formation depending on tumor type and molecular context; the receptor tyrosine kinase-like orphan receptor 2 (ROR2), a transmembrane protein that participates in Wnt signaling, is frequently silenced by promoter hypermethylation in human colon cancer; instead in osteosarcoma cells suppressed expression of ROR2 inhibits cell invasiveness (Lara *et al.*, 2010). SPARCL1 protein was over-expressed in the early stages and weakly expressed in the metastatic tumors; however, in a study of non-small cell lung carcinoma, SPARCL1 mRNA levels were found to be down-regulate. SPARCL1 protein could therefore play a dual role of oncogene in colorectal carcinoma and tumor suppressor in lung cancer (Zhang *et al.*, 2011). The discovery of methylation marks in CRC was considered of fundamental importance to correlate methylation status of specific genes with early diagnosis and prognosis. For instance, it was shown that aberrant methylation of *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes is associated to tumor initiation, but not to tumor progression (Lee *et al.*, 2009). Several epigenetically silenced genes have an important role in colorectal carcinogenesis; *APC*2, a homolog of *APC*1 tumor suppressor gene involved in Wnt signaling pathway, showed a frequency of 95.5% methylation in its promoter. ECAD, a Ca+-dependent

adhesion molecule that mediates intercellular contacts, was hypermethylated in 40.8% of primary CRCs. Loss of ECAD expression is then associated with the invasion and metastasis (Naghibalhossaini *et al.*, 2011). Lind and colleagues (Lind *et al.*, 2011) found promoter hypermethylation of the *CNRIP1*, *FBN1*, *INA*, *MAL*, *SNCA*, and *SPG20* genes was frequent in both CRCs (65–94%) and adenomas (35–91%), whereas normal mucosa samples were rarely (0–5%) methylated; the sensitivity of at least two positives among the six markers was 94% for CRCs and 93% for adenoma samples, with a specificity of 98%. Methylation of *IGFBP3, EVL, FLNC,* and *CD109* genes is associated with an eightfold increase in mortality risk relative to that of patients with no DNA methylation of these genes (Carmona *et al.*, 2011). Moreover, hypermethylation of *CDH13* and *FLBN3* genes is associated with poor prognosis in CRC (Wang *et al.*, 2011). Looking for novel prognostic biomarkers for CRC, recent studies showed that tumors that have silenced genes in the extracellular matrix remodeling pathway show worse survival (Carmona *et al.*, 2011; Yi *et al.*, 2011). Invasive screening modalities, including colonoscopy, are not ideal for application to the asymptomatic population. Therefore, active investigations are now underway to discover noninvasive biomarkers, such as those found in stool, which could supplement or supplant colonoscopic screening. DNA methylation changes have also been observed in plasma as a marker of circulating tumor DNA. Less is known about changes in WBC DNA methylation levels and cancer risk, although studies are rapidly emerging. Several studies measuring overall WBC global DNA methylation in different cancer types including colon, bladder, stomach, breast, head and neck cancer have found an elevated risk (sometimes statistically significant) for cancer between those in the lowest quantile of global DNA methylation compared to those in the highest quantile (Terry *et al.*, 2011). In plasma, cell-free methylated DNA AQ3 has been reported to be a useful biomarker of

noninvasive blood screening for the detection of CRC. Septin 9 (*SEPT9*) and vimentin (*VIM*) genes have been analyzed in blood/serum samples and stool samples and reported sensitivity and specificity range of 68–77% and 83–94%, respectively (Chen *et al.*, 2005; Devos *et al.*, 2009). Among genes that have been proven to be promising for an early diagnosis of CRC, besides *SEPT9* (Grützmann *et al.*, 2008), there are also *ALX4* and *TMEFF2* (He *et al.*, 2010). Loss of *SMAD4*, a tumor suppressor gene frequently inactivated in pancreatic and CRCs, correlates significantly with decreased survival in colon cancer patients. High *SMAD4* expression, however, is significantly associated with increased survival, especially in colon cancer patients who has undergone potential curative surgery (Isaksson-Mettävainio *et al.*, 2011). Figure 4 shows some genes methylated during the different stages of carcinogenesis and figure 5 shows the most studied genes which have been found methylated in CRC, with their possible functions in cancer initiation and progression.

NORMAL EPITHELIUM

MGMT , *P16* (respectively methylated in 20% 18% of samples); MSP assay (Krakowczyk et al., 2008)

hMLH1 (all methylation negative samples with less then 20% of methylation); PYROSEQUENCING assay (Gay et al., 2011)

hMLH1, hMSH2, MGMT (respectively methylated in 0%, 5.4%, 10.7% of samples); MSP assay (Lee et al., 2011)

Methylated EVL/hsa-miR-342 in 12% of normal mucosa from cancer-free controls and 56% of histologically normal colorectal mucosa from individuals with concurrent colorectal adenocarcinoma ('field defect'); MSP assay (Grady et al., 2008)

Extensive (including two methylation-sensitive regions) and partial (one of two regions) SFRP2 methylation levels were in 3.9 % and 39.9 % of normal colonic mucosa from CRC patients (N-Cs), and in 0 % and 30.6 % of colonic mucosa from subjects with no evidence of colorectal neoplasia at colonoscopy; COBRA (Takeda et al., 2011) .

Adenomatous polyps

The highest methylation detected was for *MGMT* gene (47.1%) followed by 35.3% for HIC-1 and 5.9% for *RASSF1A* gene; MSP assay (Abouzeid et al., 2011)

Extensive (including two methylation-sensitive regions) and partial (one of two regions) SFRP2 methylation levels were in 8.7 % and 37.9 % of samples; COBRA (Takeda et al., 2011) .

ADENOMA

hMLH1, *hMSH2* and *MGMT* respectively methylated in 1.8%, 8.0%, 33.9% of salmples; MSP assay (Lee et al., 2011)

67% of samples had methylation in EVL/hsa-miR-342; MSP assay (Grady et al., 2008)

CANCER

MGMT, P16 (respectively methylated in 59%, 53% of samples); (MSP) (Krakowczyk et al., 2008)

hMLH1 (12,3 % methylated samples with more then 20% of methylation and loss of expression); samples principally MSI-H and of proximal tumor site (PYROSEQUENCING) (Gay et al., 2011)

High frequency of methylation at *MGMT*, *RASSFA*, and *HIC-1* (respectively in 25%, 47.2%, and 41.7% of samples); MSP assay (Abouzeid et al., 2011).

86% of samples methylated in EVL/hsa-miR-342; MSP assay (Grady et al., 2008)

Extensive (including two methylation-sensitive regions) and partial (one of two regions) *SFRP2* methylation levels found in 61.7% and 24.8% of samples; COBRA (Takeda et al., 2011) .

METASTATIC CANCER

hMLH1, *MSH2*, *MGMT* (respectively methylated in 1,8%, 13.4% and 47.3% of samples; MSP (Lee et al., 2011)

Figure 4: Methylation of genes during CRC stages (Migheli and Migliore, 2012).

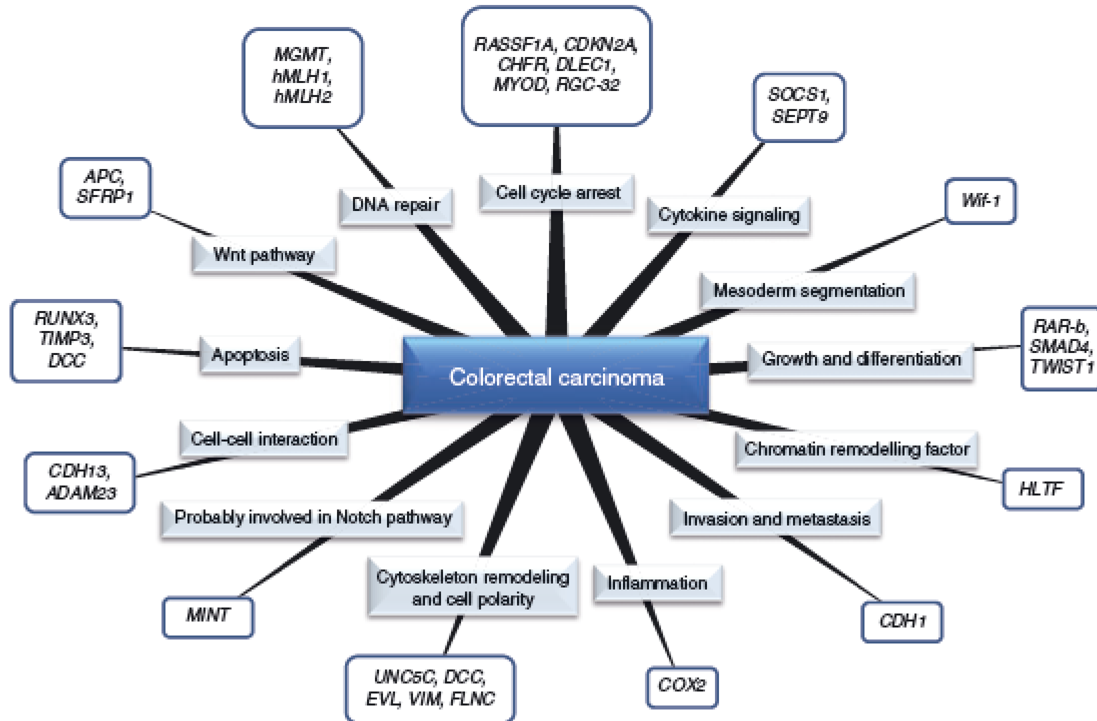


Figure 5: The methylation pattern of many genes from normal and tumor colonic mucosa from CRC patients has been extensively studied. Here we report the most studied genes and the pathways in which they are involved (Migheli and Migliore, 2012).

1.4.2 Histone modifications in colorectal cancer

Little is known about patterns of histone modification alteration in human tumors and even less in CRC. Chromatin remodeling is, together with methylation, a key mechanism for gene regulation and consists of modifications at conserved lysine residues on the tails of histone proteins; lysine acetylation allows the transcription by weakening the association of the histone with DNA and allows transcription factor binding. Lysine methylation can be associated with both active and repressed regions of DNA; trimethylation of histone H3 lysine 4 active transcription, instead methylation of H3K9 and H3K27 appears at transcriptionally silent gene promoters (Jones *et al.*, 2007). Hypomethylation alone cannot turn on silenced genes, instead increased Histone H3 acetylation with localized hypomethylation allows long-term reversion of

epigenetically silenced genes; a study showed as *CDO1, HSPC105* and *MAGEA3* were still expressed 10 days post 5-aza-dC treatment, in fact they had localized hypomethylation at the transcriptional start site and an increased histone H3 acetylation (Mossman *et al.*, 2011). Enzymes for chromatin remodeling can alter chromatin by covalent modification of histone or by using the energy from ATP hydrolysis. Included in the ATP-dependent chromatin remodeling enzyme family is the chromodomain helicase DNA-binding protein (CHD) family, which consists of nine proteins (CHD1–9) in humans. With regard to cancer, CHD5 controls proliferation and apoptosis. In humans, CHD5 is inactivated not only by deletion but also by hypermethylation. These alterations might contribute to cancer pathogenesis by deregulating CHD-mediated chromatin remodeling (Kim *et al.*, 2011). *SOCSs* and *SHP1* genes seem to have a role as tumor suppressor and DNA methylation as well as histone acetylation/deacetylation could control their transcriptional regulation in CRC cells. Xiong and colleagues (Xiong *et al.*, 2012) showed that TSA, an histone deacetylase inhibitor (HDACi), increased the mRNA levels of SOCS1 and SOCS3. The induction of SOCS1 and SOCS3 expression by TSA in human CRC cells was because of an increase in the acetylation of H3 and H4 histone proteins associated with their promoter regions. However, they did not observe any significant changes in histone acetylation of *SHP1* promoter regions. Two different studies reported that the global levels of H4K12ac and H3K18ac increased in adenocarcinomas respect to the normal tissue or adenomas and that the H3K9me2 expression was also associated with the progression adenoma–adenocarcinoma (Ashktorab *et al.*, 2009; Nakazawa *et al.*, 2011).

1.4.3 NcRNA alterations in colorectal cancer

Chromosome anomalies (deletions, translocations, copy-number alterations), DNA mutations and epigenetic deregulation of the ncRNAs or of the genes involved in their biogenesis have been described in tumor progression and the best-characterized miRNAs dysregulated by DNA hypermethylation in tumors, including CRC and the functional consequences in tumoral cells, have been reviewed recently (Lopez-Serra *et al.*, 2011). In Table 2 some recent examples of ncRNAs found dysregulated in CRC cells are shown. For instance, epigenetic alteration of miR-143 which targets *KRAS* can interfere with its expression and induce cell proliferation (Liu *et al.*, 2010); epigenetic alteration of miR-148b which targets cholecystokinin-2 receptor gene (CCK2R) can lead to cell proliferation (Song *et al.*, 2011). A small part of the miRNAs were up-regulated by 5-aza-2 deoxycytidine (5-aza-dC) treatment in CRC cells; however, different cell type like SW1116 and HT29 cell could have different drug sensitivity. DNA methylation analyses showed that the promoter region of the mir-345 gene was heavily methylated in HT29 cells (64.73%) and it was decreased to 35.78% after 5-aza-dC treatment, which results in a major mir-345 expression; *AQ4* overexpression of mir-345 may suppress colon cancer cell invasiveness in vitro. mir-345 might be involved in pathogenesis of CRC through downregulation of the expression of *BAG3*, one of the molecules that regulates apoptosis (Tang *et al.*, 2011). Hox transcript antisense intergenic RNA (HOTAIR) is a long ncRNA that regulates expression of multiple genes in cooperation with PRC2; HOTAIR expression levels in CRC tissues were higher than those in corresponding noncancerous tissues; its overexpression increased the invasiveness of CRC cells, then this ncRNA might play a role in promoting metastasis of CRC. Moreover, patients with high HOTAIR expression had a significantly poorer prognosis than those with low HOTAIR expression (Kogo *et al.*, 2011).

Table 2: Deregulation of ncRNAs in CRC by epigenetic, chromosome abnormalities (deletions, translocations, copy-number alterations), and DNA mutations (Migheli and Migliore, 2012).

ncRNA	Comments	Ref.
miR-34b/c, miR-9-1, miR-129-2 and R-137	Silencing of these genes was observed in CRC cell lines and in primary CRC tumour respect to normal mucosa.	Liu et al., 2010
miR-143	Regulates KRAS expression and cell proliferation	Liu et al., 2010
miR135	Suppression of APC expression and Wnt pathway activity	Liu et al., 2010
miR-34a	Act as a tumor suppressor by blocking *SIRT1;* regulates cell proliferation	Yamakuchi et al., 2008
mir-345	Downregulation of *BAG3;* involved in cell proliferation and invasion in human colorectal cancer	Tang et al., 2011
HOTAIR	Regulates expression of multiple genes in cooperation with *PRC2* Associated with CRC metastasis	Kogo et al., 2011
let-7c	Destabilizes K-RAS, MMP11 and PBX3 mRNAs; associated with CRC metastasis	Han et al., 2011
miR-148b	Regulates cholecystokinin-2 receptor gene (CCK2R) and cell proliferation	Song et al., 2011
let-7a	Regulates Np95 ICBP90 RING finger (NIRF) and cell proliferation	Wang et al., 2012
miR-499-5p	Suppression of FOXO4 and PDCD4; associated with CRC metastas	Liu et al., 2011

1.5 Colorectal adenoma-carcinoma sequence

An unresolved question related to genomic instability, which has been raised in many papers, is whether CIN arises early in tumorigenesis and initiates the adenoma-carcinoma sequence or whether it is acquired during this process and facilitates the formation of colon cancer. Michor *et al.* developed a mathematical representation of the evolutionary dynamics of colorectal tumorigenesis and found that one or two CIN genes in the genome are enough to make sure CIN emerges early (Michor *et al.*, 2005). However this view is not universally accepted: some authors are inclined to believe that CIN is acquired during tumorigenesis and facilitates progression to malignancy. Even in light of the latest knowledge, the question is still considered unresolved (see the recent review by Pino and Chung, 2010). Different tumors show various patterns of

aneuploidy. The loss of heterozygosis that occur in the first phases of the CRC cancerogenesis, as well as the alteration of methylation pattern of multiple key genes can drive the development of colorectal cancer by facilitating the acquisition of multiple tumor-associated mutations and the instability phenotype. Aberrant CpG island methylation is involved in cancer development, but it is not yet clear if it is a cause or an effect of cancer formation, which genes are methylated during the pathogenesis of individual cancers, when is the time of methylation and gene silencing, how specific methylation profiles are established, and what determines tumor typespecific methylation (Kim *et al.*, 2010). Restricted folate diet or SNPs in one-carbon metabolism, leading to a reduction of the total amounts of DNA methylation in human tumors results in hypomethylation of repetitive DNA sequences, contributing to the origin of cancer cells by generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting; moreover, hypomethylation could activate proto-oncogenes. The misincorporation of uracil into human DNA, favoured when thymidylate availability is restricted, could also increase the frequency of chromosome cleavage. On the other hand, tumor suppressor genes could gain CpG island methylation, resulting in the inactivation of these protecting proteins. Moreover epigenetic alterations could influence either cancer initiation or progression. In 1990 Fearon and Volgestein proposed a model for colorectal tumorigenesis. For this model the mutational activation of oncogenes and inactivation of tumor suppressor genes, lead to the development of CRC. These alterations generally occur in a vertical sequence that goes togheter with the clinical progression of the tumor, but probably it is more important the total accumulation of changes rather than their order. The key oncogene in this model is *K-RAS*, instead the tumor suppressor genes involved reside on chromosome 5q, 17p and 18q. Other somatic alterations, such as DNA methylation, are

involved (Leslie *et al.*, 2002). Figure 6 shows a colorectal adenoma-carcinoma sequence model.

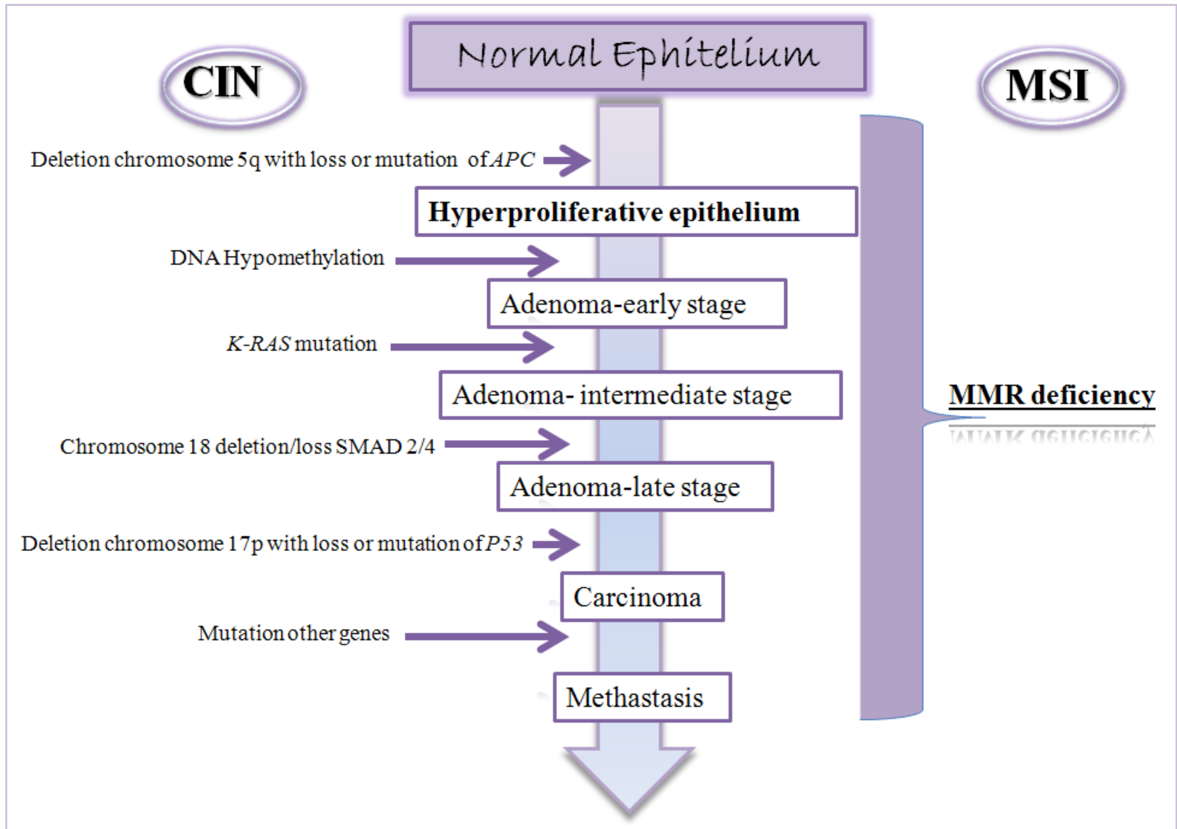
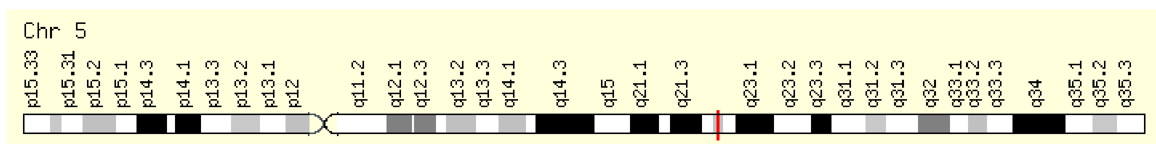


Figure 6: Colorectal adenoma-carcinoma sequence (according to Fearon and Volgestein model).

1.5.1 *APC* (Adenomatous Polyposis Coli) gene

The human *APC* gene is located on the long (q) arm of chromosome 5 between positions 21 and 22, from base pair 112,118,468 to base pair 112,209,532.



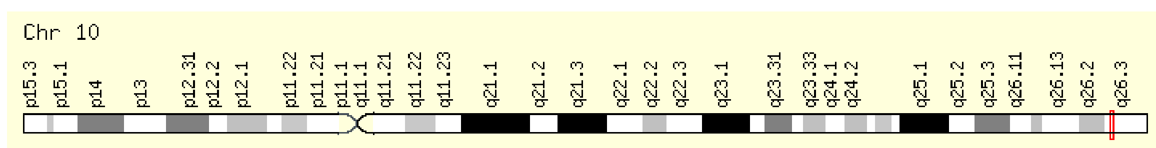
This gene encodes a tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway. It is also involved in other processes including cell migration and

adhesion, transcriptional activation, and apoptosis. Defects in this gene cause familial adenomatous polyposis (FAP), an autosomal dominant pre-malignant disease that usually progresses to malignancy (see page 5). Disease-associated mutations tend to be clustered in a small region designated the mutation cluster region (MCR) and result in a truncated protein product. *APC* activity is correlated with its phosphorylation state. The *APC* gene product (2843 amino acids; 310 kDa) indirectly regulates transcription of a number of critical cell proliferation genes, through its interaction with the transcription factor β catenin. *APC* binding to β catenin leads to ubiquitin-mediated beta catenin destruction; loss of *APC* function increases transcription of β catenin targets. These targets include cyclin D, C-myc, ephrins and caspases. Particularly the *APC* protein normally builds a complex with glycogen synthase kinase 3-beta (GSK-3β) and axin. This complex is then able to bind β- catenin in the cytoplasm; with the help of casein kinase 1 (CK1), which carries out an initial phosphorylation of β-catenin, GSK-3β is able to phosphorylate β-catenin a second time. This targets β-catenin for ubiquitination and degradation by cellular proteosomes. This prevents it from translocating into the nucleus, where it acts as a transcription factor for proliferation genes. *APC* also interacts with numerous actin and microtubule associated proteins. *APC* itself stabilizes microtubules. Hypermethylation of *APC* promoter 1A, instead of mutations involving *APC* and beta-catenin, contributes to moderate activation of Wnt signalling in a subset of serrated adenomas (Fu *et al.*, 2009). A study analyzed the methylation status of 10 genes in fresh-frozen tissues and corresponding plasma samples from patients from stage I and II of sporadic colorectal cancer, 276 healthy individuals, and plasma from 64 colorectal adenoma patients using MSP. The methylation was detected in 18% for *p14*, 34% for $p16^{Ink4A}$, 27% for *APC*, 34% for *DAPK*, 32% for *HLTF*, 21% for h*MLH1*, 39% for *MGMT*, 24% for *RARbeta2*, 58% for *RASSF2A*, and 74% for *Wif-1*; the author

concluded that tumor-specific methylation of *APC*, *MGMT*, *RASSF2A*, and *Wif-1* genes might be a valuable biomarker in plasma for the early detection of CRC (Lee *et al.*, 2009).

1.5.2 *MGMT* (O-6-methylguanine-DNA methyltransferase) gene

Cytogenetic Location 10q26.3: Start: 131,265,448 bp from pter - End: 131,566,271 bp from pter; Size: 300,824 bases. Protein: 207 amino acids; 21646 Da.



O(6)-alkyl-guanine is the major carcinogenic lesion in DNA induced by alkylating mutagens. This DNA adduct is removed by the repair protein, O(6)-methylguanine-DNA methyltransferase. This protein is not a true enzyme since it accepts the alkyl group from the lesion in a stoichiometric reaction and the active enzyme is not regenerated after it is alkylated. The methyl-acceptor residue in the protein is cysteine. *MGMT* promoter methylation in normal colonic mucosa might be a predisposing factor for cancer as a field effect and an early event in colorectal carcinogenesis. *MGMT* promoter methylation and loss of expression have been associated with G>A mutations in a variety of genes such as *KRAS, PIK3CA*, *TP53*, and *APC* (Halford *et al.* 2005; Shen *et al.*, 2005; Ogino *et al.* 2007; Nagasaka *et al.*, 2008; Nosho *et al.* 2008). DNA hypermethylation for *hMLH1* and *MGMT* DNA repair genes was reported in precursor lesions to colorectal cancer. These epigenetic alterations may be influenced by factors such as xenoestrogens, folate, and multivitamins. Detection of these changes may help determining cancer susceptibility and early diagnosis (Dumitrescu, 2012). Promoter hypermethylation status of *RASSAF1A*, *MGMT*, and *HIC-1* genes were determined in

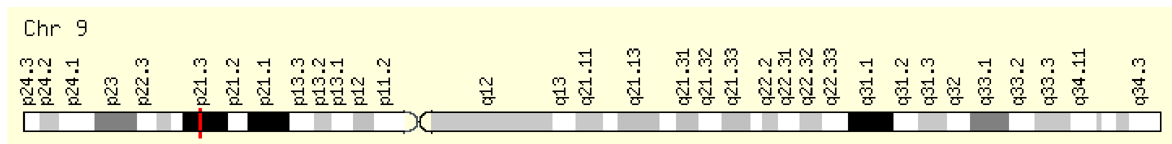
36 CRC, 17 adenomatous polyps, and 19 ulcerative colitis, and adjacent normal-appearing tissues using MSP assay. High frequency of methylation at *MGMT*, *RASSF1A*, and *HIC-1* genes was detected in CRC patients (25%, 47.2%, and 41.7% respectively). The highest methylation observed in adenomatous polyps patients was in *MGMT* gene (47.1%) followed by 35.3% for *HIC-1* gene and only 5.9% for *RASSF1A* gene; they also found an association between methylation at *RASSF1A* gene with gender ($p = 0.005$) (Abouzeid *et al.*, 2011). Morning stool specimens were collected from 69 patients with colorectal cancer, 24 with colon adenoma, 19 with hyperplastic polyps, and 26 healthy controls. The methylation frequencies of *MAL*, *CDKN2A* and *MGMT* were 78.3%, 52.5% and 55.1% in colorectal cancer, 58.3%, 41.7% and 37.5% in colon adenomas, 26.3%, 15.8% and 10.5% in hyperplastic polyps, and 3.8%, 0 and 3.8% in healthy controls, respectively. Significant differences in three genes were found between colorectal cancer and hyperplastic polyp, colorectal cancer and healthy control, colon adenoma and hyperplastic polyp, colon adenoma and healthy control (all $P<0.05$). The diagnostic sensitivity by combining three methylation markers was 92.8% in colorectal cancer, 70.8% in colon adenomas, significantly higher than fecal occult blood test (29.0% in colorectal cancer and 25.0% in colon adenomas, all $P<0.05$). No significant associations existed between methylation of the three genes and clinical characteristic including sex, age, tumor location, lymphnode metastases and TNM stage (all $P>0.05$) (Kang *et al.*, 2011). A study showed an association (81%, $\kappa = 0.59$, $p < 0.0001$) between *MGMT* methylation and MGMT loss. *MGMT* methylation and loss of MGMT were not perfectly correlated; in fact *MGMT* expression may be caused not only by promoter methylation but also by other mechanisms such as a gene mutation. Second, promoter methylation may be present in only one *MGMT* allele, and the MGMT protein may be expressed from the second allele. Third, there may be other

molecules such as ncRNA, that may downregulate *MGMT* (Shima *et al.*, 2011). It was hypothesised that an *MGMT* field defect may constitute a preneoplastic event for the development of MMR-deficient tumors displaying microsatellite instability (MSI). Loss of *MGMT* expression was more frequent in MSI than MSS CRC (p=0.047); moreover loss of *MGMT* expression was associated with *MGMT* gene promoter methylation (p=0.03) (Svrcek *et al.*, 2010). Methylation status of the *MGMT* gene was examined in primary carcinomas and the corresponding normal tissues in 48 patients with CRC using MSP; aberrant methylation was detected in 21% of primary colorectal cancers. There was no statistically significant association between abnormal methylation and gender or age, maximal tumor size, tumor extent, tumor site, histology, lymphnode metastasis, and TNM stage. All stages of colorectal cancers presented *MGMT* methylation, indicating that the *MGMT* gene has been methylated from the early stages of colorectal cancers (Hibi *et al.*, 2009). A study observed the incidence of *MGMT* methylation increased along the adenoma–carcinoma sequence, and the evaluation of the contribution of *MGMT* methylation to the development of an advanced lesion was statistically significant. The increased incidence of *MGMT* methylation in normal-appearing mucosa could represent the “field defect”. Authors reported that *MGMT* promoter methylation occurred frequently in the apparently normal colonic mucosa of CRC patients with tumors that also showed *MGMT* promoter methylation; in contrast, *MGMT* promoter methylation was observed much less frequently in healthy subjects and in the apparently normal colonic mucosa of CRC patients with tumors that did not show *MGMT* methylation. In addition, they observed an association between age and promoter methylation levels. Methylation detected by MSP may not affect the expression of gene products if methylation affects only a few cells or if the methylation

of one copy happens with an absence of methylation in the other copy of the gene (Lee *et al.*, 2011).

1.5.3 CDKN2A or $p16^{Ink4A}$ (Cyclin-dependent kinase inhibitor 2A) gene

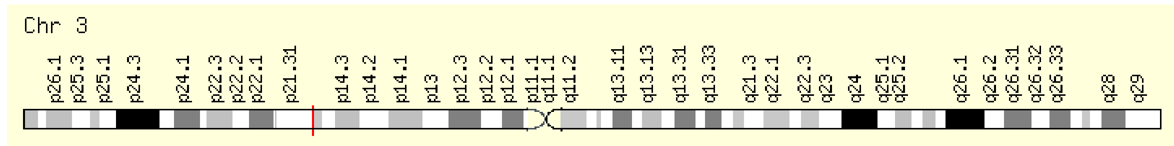
Cytogenetic Location 9p21.3: Start: 21,967,751 bp from pter- End: 21,995,300 bp from pter; Size: 27,550 bases.



This gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. It acts as a tumor suppressor and has to arrest the cell cycle in G1 and G2 phases. It binds to MDM2 and inhibits its oncogenic action by blocking MDM2-induced degradation of p53 and enhancing p53-dependent transactivation and apoptosis. It also induces G2 arrest and apoptosis in a p53-independent manner by preventing the activation of cyclin B1/CDC2 complexes. Increased expression of the $p16^{Ink4A}$ gene reduces the proliferation of stem cells. This reduction in the division and production of stem cells protects against cancer while increases the risks associated with cellular senescence. Analysis of $p16^{Ink4A}$ promoter methylation was performed by MSP assay. 42.1% of the CRC were found to have the $p16^{Ink4A}$ gene methylated. The methylation status was found to be associated with the gender, lymph node status, tumor stage, smoking status and tumor grade of the CRC patients. $P16^{INK4A}$ plays a pivotal role in tumor development and progression to advanced stages (Sameer *et al.*, 2012). $P16^{INK4A}$ methylation was evaluated by Q-MSP technique in the serum of CRC patients during

their follow-up period. The *p16*^{Ink4A} methylation decreased during two weeks after surgery. One month after surgery, in the patients with recurrence of cancer, a great increase in *p16*^{Ink4A} methylation was observed, while in the disease-free patients no methylation was seen more; so methylation could sensitively reflect the recurrence status and may be useful for identifying the presence of recurrence during the follow-up of CRC patients (Nakayama *et al.*, 2011). Colorectal cancer (CRC) screening using stool DNA has yielded a greater detection rate than conventional fecal occult blood testing. Stool samples from 31 healthy controls, 25 patients with adenomas and 30 patients with CRC were analyzed and methylation regard to *ITGA4*, *SFRP2* and *p16*^{Ink4A} promoters were observed in 36.7%, 60.0%, and 40.0% of the CRC samples and in 16.0%, 44.0%, and 24.0% of the colorectal adenomas, respectively. The methylation status had high sensitivity and specificity for the detection of colorectal adenomas and CRC; thus stool screening might be a useful non-invasive method for CRC detection (Chang *et al.*, 2010). Aberrant methylation of *p16*^{Ink4A} and h*MLH1* promoters was found in 47.2% and 53.4% of tumors respectively. For adjacent normal mucosa, 30% of patients were fully unmethylated in *p16*^{Ink4A} promoter, whereas h*MLH1* promoter was predominantly unmethylated (76%). Methylation of *p16*^{Ink4AI} correlated with gender and tumor size, whereas that of h*MLH1* significantly correlated with overall survival. Concomitant methylation of *CDKN2A* and h*MLH1* genes was associated with TNM stage and tumor size (Miladi-Abdennadher *et al.*, 2011).

1.5.4 RASSF1A (Ras association (RalGDS/AF-6) domain family member 1) gene



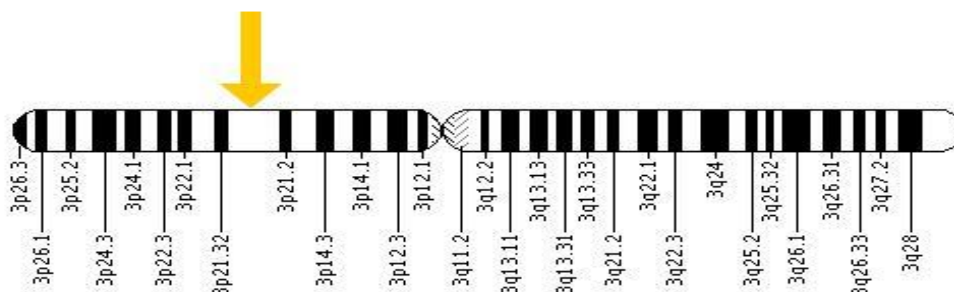
Cytogenetic Location 3p21.31: Start: 50,367,217 bp from pter-End: 50,378,411 bp from pter; Size: 11,195 bases.

Several alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported. It is a potential tumor suppressor. Isoform A interacts with CDC20, an activator of the anaphase-promoting complex (APC^{Cdc20}) that initiates chromatid separation and entrance into anaphase. The APC^{Cdc20} protein complex targets securin for destruction, enabling sister chromatid separation. It also targets S and M-phase (S/M) cyclins for destruction, which inactivates S/M cyclin-dependent kinases (Cdks) and allows the cell to exit from mitosis. Isoform A also disrupts interactions among MDM2, DAXX and USP7, thus contributing to the efficient activation of *TP53* by promoting MDM2 self-ubiquitination in cell-cycle checkpoint control in response to DNA damage. MSP was used to examine the promoter methylation status of the serum *RASSF1A* gene; the *RASSF1A* promoter hypermethylation in gastric (34.0%) and colorectal (28.9%) adenocarcinoma patients were significantly higher than those in patients with benign gastric (3.3%) or colorectal (6.7%) disease or in healthy donors (0%) ($P < 0.01$). Although the serum *RASSF1A* promoter hypermethylation frequency tended to be higher in patients with distant metastases, there was no correlation between methylation status and metastasis. There was no correlation between serum *RASSF1A* promoter hypermethylation and sex, age, tumor differentiation grade, surgical therapy, or serum carcinoembryonic antigen level (Wang *et al.*, 2008). A study analyzed, by mean of Q-MSP, primary tumor and synchronous liver metastatic tissues of 75 CRC patients and

evaluated *K-RAS* and *BRAF* mutations. *RARbeta*, *RASSF1A*, and *CDKN2A* genes were methylated in 82%, 35%, and 26% of primary tumors, respectively. *RASSF1A* methylation status was significantly higher in liver metastasis with respect to primary tumor (P=0.000) underlying the role of this gene in liver metastatic progression. Moreover *K-RAS* and *BRAF* were mutated in 39% and 4% of cases, respectively and *RASSF1A* methylation resulted significantly higher in liver than in primary tumor (P=0.05) only in *K-RAS* wild-type patients. All Ras protein family members are GTPase; when *RAS* is 'switched on' by incoming signals, it subsequently switches on other proteins, which turn on genes involved in cell growth, differentiation and survival. As a result, mutations in *RAS* genes can lead to the production of permanently activated Ras proteins. This can cause unintended and overactive signaling inside the cell, even in the absence of incoming signals. Ras is the most common oncogene in human cancer; mutations that permanently activate Ras are found in 20-25% of all human tumors and up to 90% in certain types of cancer (Tommasi *et al.*, 2011).

1.5.5 *hMLH1* (mutL homolog 1, colon cancer, nonpolyposis type 2) gene

Cytogenetic Location: 3p21.3; from 37,034,840 to base pair 37,092,336; The *hMLH1* gene is composed of 19 exons spanning in a region of 57496 bp. Protein: Aminoacids: 756. Molecular Weight: 84.6 kDa.



The *MLH1* gene is a member of a set of genes known as the mismatch repair (MMR) genes. This protein fixes mistakes that are made during DNA replication in preparation for cell division. It contains an ATPase domain and two interaction domains, one for MutS homologs (MSH2, MSH3, MSH6) and the other for PMS2, MLH3 or PMS1. MLH1 has no known enzymatic activity. MLH1 forms a heterodimer with PMS2 known as MutLa, although it can also bind to PMS1 or MLH3. This heterodimeric complex binds to the heteroduplexes MutSa (composed of MSH2 and MSH6) or MutSb (composed of MSH2 and MSH3), which recognizes DNA lesions. The heterodimer formed by MLH1 is responsible for the recruitment of the proteins needed for the excision and repair synthesis. The repair is made by removing a section of DNA that contains mistakes and replacing the section with a corrected DNA sequence. A study evaluated the frequency of promoter methylation for *hMLH1*, *hMSH2,* and *MGMT* in colorectal non-tumoral mucosa adenoma, and adenocarcinoma by mean MSP assay. The *hMLH1* promoter was methylated in 1.8% of the adenoma and adenocarcinoma samples. No methylation of *hMLH1* was found in any corresponding non-tumoral mucosa tested. *MSH2* promoter methylation was found in 8.0% of adenoma and 13.4% of adenocarcinoma samples. *MGMT* methylation was detected in 33.9% of adenoma and 47.3% of carcinoma samples. In the corresponding normal-appearing mucosa, methylation was seen in *hMSH2* (5.4%) and *MGMT* (10.7%), but not in *hMLH1* (Lee *et al.*, 2011). In other study by Psofaki *et al.* (2010) promoter *hMLH1* hypermethylation was observed in tumor samples, but not in blood samples.

1.6 Environmental factors that might influence epigenetic patterns in colorectal cancer.

Studies where people migrate from low to high CRC risk areas of the world, demonstrate that changes of diet and physical activity enhance the incidence of cancer in a high-risk country even over one or two generations (Nystrom *et al.*, 2009). Considering CpG island methylation levels in normal colorectal mucosa of different racial groups, some Authors found that African Americans had lower levels of methylation compared to Caucasians and Hispanics. Possible explanations for these findings, include lifestyle factors, genetic protection or predisposition to methylation (Wallace *et al.*, 2010). Moreover, monozygotic twins carriers of high penetrant genetic alteration in HNPCC, associated with *hMLH1* mutations, develop cancer at different ages. These observations suggest a role of the environment in epigenetic changes (Esteller *et al.*, 2008).

It was observed that a positive association between vitamin B6 and rectal cancer risk exists in women. Among men, methionine was associated with a decreased risk of proximal colon cancer whilst among women it was inversely associated with rectal cancer (De Vogel *et al.*, 2008).

Choline could derive from the diet, but also from de novo biosynthesis by means of an enzyme coded by the gene phosphatidylethanolamine-N-methyltransferase (*PEMT*). One of the choline metabolites, betaine, participates in the methylation of homocysteine to form methionine. Then choline and betaine have been hypothesized to decrease the risk of colorectal cancer. Estrogens cause a marked upregulation in PMET mRNA expression and enzyme activity, then premenopausal women have an enhanced capacity for de novo biosynthesis of choline. In choline deficient cells in culture, and in fetal

rodent brains from mothers fed with choline-deficient diets, methylation of the *CDKN3* gene promoter decreased, resulting in overexpression of this gene which inhibits cell proliferation. Maternal diet high in choline and/or methionine and/or methyl-folate during pregnancy results in epigenetic changes in gene expression in the fetus (Zeisel *et al.*, 2007).

Alcohol consumption was found to be a risk factor for colorectal tumorigenesis. In an in vivo study (male rats), it was observed that a decrease in RFC1 (reduced folate carrier) mRNA and protein expression correlates with alcoholism. That is a possible reason of lower blood folate levels commonly found in chronic alcoholics (Hamid *et al.*, 2009). Alcohol in murine studies appears to reduce MTR levels; thus it could induce DNA hypomethylation (Arasaradnam *et al.*, 2008). Alcohol consumption may in fact decrease DNA methylation in hepatic tissue by affecting folate metabolism and/or methionine synthesis, which might decrease levels of the S-adenosylmethionine (SAM), the main methyl donor of DNA. Excessive alcohol consumption has been shown to alter DNA methylation in humans. Patients suffering from alcoholism had 8–10% higher methylation in CCGG sequences respect to controls. Associations between alcohol drinking and DNA repetitive element methylation levels have shown inverse associations between Alu and alcohol drinking. However, several studies did not find an association between alcohol consumption and LINE1 methylation. One study found a significant increase in *HERP* (homocysteine-induced ER protein) promoter methylation in patients with alcohol dependence (Terry *et al.*, 2011). Moreover in a Dutch study, higher frequency of promoter methylation of *APC1A*, *P14*, *hMLH1*, *MGMT*, and *RASSF1A* genes was observed in presence of a low-folate/high-alcohol diet.

Tobacco could also influence CRC risk. The methylation levels for *MGMT*, RAR-b, and SST decrease in the following sequence: nonsmokers without colorectal adenomas > smokers without colorectal adenomas > nonsmokers with colorectal adenomas > smokers with colorectal adenomas. Smoking predisposes to diminished methylation of several genes, which, in turn, contribute to colorectal adenoma development (Paun *et al.*, 2010). A study reported that the risk of CIMP-high tumor, among smokers of 20 or more cigarettes per day, was higher among those with low folate and low fiber intake and those who had greater long-term alcohol consumption, although without statistical significance. Moreover, among women alcohol and cigarette smoking were associated with risk of CIMP-high tumors; women who smoked 20 or more cigarettes per day and consumed little or no alcohol did not have an increased risk of a CIMP-high tumors (Slattery *et al.*, 2007). The cellular modifications due to exposures to the chemicals present in cigarette smoke have been widely investigated and include DNA adducts, gene mutations, micronuclei, chromosome aberrations, sister chromatin exchanges and DNA strand breaks; DNMTs seem to bind DNA damage sites, which results in altered methylation patterns on these regions, suggesting a molecular mechanism for the generation of aberrant DNA methylation by exposure to chemicals such as those present in cigarettes. Only a few studies have investigated overall 5-mC content and smoking, and found no difference by cigarette smoking patterns. More research has focused on the effect of smoking on the methylation levels of repetitive elements. LINE-1, Alu and AluYb8 methylation did not differ between current and never/former smokers. It has been suggested that chemicals affecting epigenetic marks could have a larger impact when exposure occurs in utero or earlier in life, a time when epigenetic modifications are being established. Overall levels of genomic DNA methylation have been investigated in several studies in children and adults. Prenatal exposure to cigarette

smoke has been associated with an increase in the overall blood levels of DNA methylation in adulthood. LINE-1, Sat2 and Alu methylation was analyzed in adults and children prenatally exposed to smoking. LINE-1 and Sat2 levels were lower in exposed individuals when compared to unexposed ones. However, other elements such as LINE-1 do not seem to be affected by exposure to cigarette smoke. Several genes were investigated in blood samples from adults in a cohort of individuals born preterm that were exposed to smoking, with lower levels found for *IGF2* methylation (see review by Terry *et al.*, 2011).

In a study in vitro on HT 29 cells, green tea was found to inhibite DNMT1 causing CpG demethylation and reactivation of previously methylated genes (h*MLH1*, *MGMT*, $P16^{INK4A}$). There is strong evidence that polyphenols from tea, soft fruits and berries, vegetables, apples, and even from wine, are potent anti-carcinogenic agents in vitro and in animal models that prevent DNA instability. Several polyphenols are potent inhibitors of DNMT activity in vitro, capable of reversing DNA hypermethylation and reactivating tumor suppressor genes activity. Polyphenols inhibit DNMT activity and DNA methylation in two ways: by direct insertion into the binding pocket of DNMT (competitive inhibition) or indirectly by decreasing intracellular SAM concentrations. Tea components (especially green and black tea) and specific soya isoflavones inhibit DNMT activity in human cancer cells. DNMT 1 inhibition demethylates CpG islands in the promoter regions of silenced tumor suppressor genes including $p16^{Ink4A}$, retinoic acid receptor b, *MGMT*, m*MLH1* and GST. Green tea (EGCG) can reactivate retinoic acid receptor b in prostate and breast cancer cells, $p16^{Ink4A}$ in colon cancer cells and GST in prostate cells. Genistein could reverse DNA hypermethylation and reactivate $p16^{Ink4A}$, retinoic acid receptor b and *MGMT* gene expression (Duthie, 2011).

Also selenium suppressed aberrant DNA methylation by means of DNMT inhibition (Arasaradnam *et al.*, 2008).

Arsenic has been associated with gene-specific hypermethylation of *p53* and $p16^{Ink4A}$ promoter regions in blood DNA of subjects exposed to toxic level of arsenic compared to controls. Hypermethylation of the $p16^{Ink4A}$ promoter was also observed in WBC DNA from 103 patients with arseniasis compared to 110 healthy subjects (see review Terry *et al.*, 2011).

Fiber intake reduces CRC risk, with dilution of fecal potential carcinogens, promoting a favorable colonic microflora, by adsorption of bile acids and by production of protective short-chain fatty acids (SCFAs principally acetate, propionate, and butyrate) through fermentation by endosymbiotic bacteria. Butyrate is a potent inhibitor of histone deacetylases (HDACs); reduced levels of butyrate will cause alterations in global protein acetylation, which may be permissive for colorectal cancer progression, while elevation of fibre levels and consequent butyrate levels may reduce or reverse these processes and restore a “normal” profile of protein acetylation. Acetyl proteins identified are nuclear structural proteins, transcription factors including p53, Sp1, and Sp3, and structural proteins including tubulin and cytokeratins (Corfe *et al.*, 2009). Higher dietary fiber assumption was associated with reduced risk of having a CIMP-high tumor (Slattery *et al.*, 2007).

DNA methylation has been also hypothesized as being a consequence of inflammation. Prostaglandins, that promote inflammation and fever, are produced within the body cells by the enzyme cyclooxygenase (COX). Nonsteroidal anti-inflammatory drugs

(NSAIDs) block the COX enzymes and reduce prostaglandins throughout the body, then their assumption has been hypothesized to be associated with development of colon tumors that display CIMP. Physical activity and use of NSAIDs were inversely associated with both CIMP-low and CIMP-high tumors. The protective effect associated with regular NSAIDs use largely disappeared among heavy smokers (Slattery *et al.*, 2007). Using a cross-sectional design, Zhang and colleagues reported a trend of higher levels of LINE-1 methylation with higher levels of physical activity (Zhang *et al.*, 2011a).

Body mass index Several studies investigating LINE-1 and Alu methylation levels were not correlated with BMI; in contrast, in a study of women of child bearing age, Piyathilake *et al.* (2011) found that higher BMI was associated with lower LINE-1 methylation. Obese individuals were at 2-fold increased risk of CIMP-low colon cancer, but obesity does not influence CIMP-high tumors (Slattery *et al.*, 2007).

Energy restriction during adolescence and early adulthood is associated with the CIMP phenotype in CRC, suggesting that exposure to a transient environmental condition during this period of life may result in persistent epigenetic changes that later influence CRC development. Individuals exposed to a period of severe short-term energy restriction had a decreased risk of developing a CIMP tumor later in life respect to individuals who were not exposed. During the years with a rapid growth and hormonal change, insulin-like growth factor-1 (IGF-1) can be four-times the normal adult serum concentration. IGF-1 inhibits apoptosis and stimulates proliferation of colonic epithelial cells in vitro. High levels of IGF-1 have been associated with a significant increase in colon cancer risk (Olivo-Marston *et al.*, 2009). It could be possible that energy

restriction, during puberty, may permanently influence the growth hormone-IGF axis, subsequently influencing methylation patterns later in life (Hughes *et al.*, 2009).

Some studies in humans evaluated the association between DNA methylation and other environmental factors such as benzene, persistent organic pollutants, and air contaminants. Benzene: Low-dose benzene exposure was associated with decreased methylation of LINE-1 and Alu sequences, and with hypermethylation of p15 and hypomethylation of MAGE-1. In patients with benzene poisoning, the average methylation level of $p16^{Ink4A}$ was higher respect to controls.

Persistent organic pollutants: Some studies investigate the correlation between plasma persistent organic pollutant (POP) concentrations and WBC global DNA methylation and found a significant inverse linear relationship for DDT, DDE, β-hexachlorocyclohexane, oxychlordane, α-chlordane, mirex, several PCBs and the sum of all persistent organic pollutants with Alu methylation. A study in Korea found that some pesticides were inversely and significantly associated with Alu methylation; however there are still contrasting results (Rusiecki *et al.*, 2008).

Air pollution. The effects of particulate matter (PM) exposure on Alu, LINE-1 and gene-specific methylation was examined in steel plant workers. Long-term exposure to PM10 was negatively associated with methylation in both Alu and LINE-1. Exposure to black carbon (BC), a marker of traffic particles, was also associated with decreased DNA methylation in LINE-1. NOS2 promoter methylation was significantly lower in post-exposure blood samples compared to baseline (Madrigano *et al.*, 2011; Tarantini *et al.*, 2009).

Folate metabolism, also known as one-carbon metabolism, is fundamental for the synthesis of DNA and RNA precursors or for the conversion of homocysteine (Hcy) to methionine, which is then used to form the main DNA methylating agent S-adenosylmethionine. Folic acid supplementation might protect the developing colorectum because of its critical role in maintaining DNA stability. In vitro animal and human studies show that folate deficiency induces epigenetic changes leading to global DNA hypomethylation, protooncogene activation and CIN; moreover, folate provide the nucleotide precursors for DNA synthesis and replication, ensuring DNA fidelity, maintenance of DNA integrity, and optimal DNA repair. In the normal colorectum, folate deficiency appears to enhance, whereas folic acid supplementation suppresses, the development of CRC. In contrast, once aberrant crypt foci are established, folate deficiency inhibits the progression and induces regression of these established pre-neoplastic foci (Kim, 2007; Kim, 2008). A deficiency of methyl groups in the diet might lead to hypomethylation of DNA in several tissues, including blood. Global and p53 gene DNA was hypomethylated in human colon adenoma cells grown in folate-depleted medium but was restored by folic acid repletion (Wasson *et al.*, 2006). Lymphocyte DNA is hypomethylated in women made experimentally folate deficient over several weeks (Jacob *et al.*, 1998; Rampersaud *et al.*, 2000) and low-dietary folate intake (<200 mg/d) correlates with hypomethylation of LINE-1 in human colon tumors (Schernhammer *et al.*, 2010). Several studies, using rat models of CRC, showed that the maternal folic acid supplementation significantly reduced the CRC risk by 64% in the offspring, whereas post weaning folic acid supplementation had no effect; moreover, tumor multiplicity were significantly higher in the pups from the dams fed the control diet and with post weaning folic acid supplementation than those without post weaning supplementation; it has been hypothesized that the pups from the dams with control diet

have developed precancerous lesions in the colorectum respect to those from the folic acid-supplemented dams; then post weaning folic acid supplementation might have promoted the progression of these pre-neoplastic lesions (Lindzon *et al.*, 2009; Sie *et al.*, 2011). Studies carried out in United States and Chile suggest that increased intake of synthetic folic acid has increased colon cancer risk (Mason *et al.*, 2007; Hirsch *et al.*, 2009). However, low dietary folate intake (<200 μg/day) was also associated with an increased frequency of hypomethylated long-interspersed nucleotide element (a marker of genome-wide DNA methylation) repeats in human colon tumors (Schernhammer *et al.*, 2010). Folate deficiency significantly increased hepatic OGG-1 and MGMT repair activity. Probably the upregulation of these two proteins indicates the occurrence of DNA damage lending further support to the finding that increased DNA damage (including DNA strand breaks, uracil misincorporation and oxidised bases) is a consequence of folate deficiency. Low folate intake significantly increased 8-oxo-7,8-dihydroguanine levels in DNA in lymphocytes from rats fed the folate deficient diet. Although there were highly significant changes in *OGG-1* and *MGMT* expression in rat liver in response to folate depletion, no such effects were seen in colon, indicating that the ability of the liver to respond to folate deficiency is not shared by the colon. The colon cannot respond such as the liver to damage and would therefore be more susceptible to the genotoxic effects instigated by folate deficiency (Duthie *et al.*, 2010). A key question is whether the findings from these animal studies can be extrapolated to humans. Little is known currently about the effect of folate status on DNA repair in humans. NER is impaired in lymphocytes from individuals with poor folate status (Wei *et al.*, 2003). Conversely, supplementing healthy volunteers of adequate folate status with folic acid (1.2mg/day for 12 weeks) does not alter BER-mediated excision of 8-oxo-7,8-dihydroguanine from lymphocytes (Basten *et al.*, 2006). They have also shown

that MGMT activity in normal human colorectal mucosa is inversely associated with vegetable consumption and that high dietary folate intake is related to low DNA alkylation damage. If this inability of colon tissue to upregulate DNA repair processes occurs in humans, it may constitute one of the mechanism through which folate deficiency increases the potential for malignant transformation (Duthie *et al.*, 2010). Low levels of folate in the diet or in blood were associated with higher CRC risk; inversely high intake of folate has been associated with reduced CRC risk (Du *et al.*, 2010). It also observed that high plasma folate levels may be associated with increased CRC risk (Van Guelpen *et al.*, 2006). In a study a small trend for higher levels of serum folate was observed in the group of patients with methylated tumors, compared to those with unmethylated tumors; moreover it was observed a trend for association between serum folate/vitamin B12 levels and gene promoter methylation: higher serumfolate/vitamin B12 levels were strongly associated with promoter methylation of $p16^{Ink4A}$ and had an association trend with promoter methylation of *MLH1* and *MLH2* genes (Mokarram *et al.*, 2009). RBC folate levels were found positively associated with *ERα* and *SFRP1* methylation levels. These data have important implications regarding the safety of supplementary folate administration in healthy adults, given the hypothesis that methylation in normal mucosa may be a predisposing phenomenon for colorectal neoplasia (Wallace *et al.*, 2010). Alterations in folate metabolizing genes or deficiency of folate can result in elevated homocysteine levels. Folate deficiency is significantly more associated with oncogenesis when combined with hyper-omocysteinemia (increased risk of 17 times of carcinogenic lesions); moreover, inflammatory bowel disease patients with folate deficiency and hyperhomocysteinemia might be associated with increased risk colorectal cancer (Phelip *et al.*, 2008). The effect of intervention with folic acid on DNA methylation is thereby conflicting and highly dependent on

initial folate status, level and duration of supplementation, tissues examined, stage of malignant transformation, and polymorphisms in folate metabolizing genes (Sie *et al.*, 2011).

Global DNA hypomethylation is the first epigenetic alteration that was recognized in CRC, occurring gradually, age dependently (Suzuki *et al.*, 2006). Growing evidence supports that WBC global DNA methylation, particularly in blood, changes with age. Studies have correlated age with two of the repetitive elements, LINE-1 and Alu. Although one study correlated positively lower levels of LINE-1 methylation with increasing age, most of the studies did not find an age- dependent effect on blood LINE-1 methylation. In contrast, all of the studies investigating age and Alu methylation supported a lower level of DNA methylation of this repetitive element DNA with increasing age. On the other hand hypermethylation of tumor suppressor genes could be positively correlate with age (An *et al.*, 2010).

Many studies have found that global DNA methylation was higher in males than in females. Of the repetitive elements, LINE-1 was associated with lower levels in females compared to males in most but not all studies. On the other hand there has been no consistent pattern associated with Alu methylation. A gender-dependent difference in gene-specific methylation in blood was also reported for *CALCA*, *MGMT*, *MTHFR*, *MAOA, DRD4, SERT* and *F8* genes (See review Terry *et al.*, 2011).

1.7 Genetic factors affecting DNA methylation in colorectal cancer

Folate is a fundamental nutrient mainly required for either DNA synthesis or methylation processes. Particularly, it is required for the synthesis of S-adenosylmethionine (SAM) the major intracellular methyl donor. The availability of

SAM is directly influenced by the diet. SAM is formed from methyl groups derived from choline, methionine, or methyltetrahydrofolate (MTHF). Because of their involvement in DNA methylation, single nucleotide polymorphisms (SNPs) in genes involved in folate metabolism could be associated with either aberrant gene methylation or CIMP. Indeed, several polymorphisms of genes involved in folate metabolism, including methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C, methionine synthase (*MTR*) A2756G andmethionine synthase reductase (*MTRR*) A66G; thymidylate synthase (*TYMS*) 28 bp repeats, DNA methyltransferase (*DNMT3B*) -149C>T, and the transcobalamin II (*TCNII*) 776G variant, often in combination with folate intake, have been associated with CRC risk, CIMP, MSI phenotypes, and aberrant methylation of CRC genes (Karpinski *et al.*, 2010; De Vogel *et al.*, 2009; Iacopetta *et al.*, 2009; Yamaji *et al.*, 2009; Curtin *et al.*, 2007; Eaton *et al.*, 2005; Kang *et al.*, 2005; Keku *et al.*, 2002; Levine *et al.*, 2000; Slattery *et al.*, 1999).

2. DNA methylation analysis techniques

There are many techniques used to investigate DNA methylation, including methylation specific PCR (MSP), quantitative MSP and Methylation sensitive-High Resolution Melting (MS-HRM). MSP allows the determination of the methylation status of a gene by means of two specific sets of primer, the first specific for the methylated template and the latter for the unmethylated one. It is important to include several CpG sites towards the 3'end of the primers to have a specific binding and amplification of only methylated variants of the template (in concert with stringent PCR conditions). MSP assays are normally associated with high false-positive rates, events that could happen with the mismatches of the primer sequence with the template or with the incomplete bisulfate conversion. False priming events could be prevent by a minor number of cycles and or using a higher annealing temperature. In the traditional MSP technique a second set of primers is designed for the amplification of unmethylated DNA. Gel electrophoresis is used to analyze the PCR products and to evaluate (in case in which both unmethylated DNA and methylated DNA are present in the test tube), by means of the band strengths, an approximation of the methylation status of the sample. A quantitative MSP technique is the Methylight assay. Some methylation studies use one primer set (MIP, methylation independent primer) to obtain a PCR product from bisulfite modified template regardless of its methylation status. MS-HRM combines PCR amplification using methylation independent primers with subsequent HRM analyses of the PCR products; the PCR products generated from a methylated template has a higher GC content and therefore higher melting temperature respect to PCR products obtained from an unmethylated template. This technique allows for estimation of the methylation level by comparing the melting profiles of unknown PCR products to

the melting profiles of PCR products derived from DNA standards with a known unmethylated to methylated template ratio.

Cloning and sequencing provide information about CpG methylation on the same molecule instead Pyrosequencing provides an average of the many molecules analyzed simultaneously (Tost and Gut, 2007). Pyrosequencing is a sequence by synthesis method that is the only method analyzing methylation levels at each CpG sites separately; nevertheless, this technique is much expensive. During Pyrosequencing PPi molecules are released, after incorporation of nucleotides, and are quantitatively converted into a bioluminometric signal; particularly the PPi is converted into ATP by the ATP sulfurylase, using adenosine 5' phosphosulfate as substrate. The energy obtained is necessary for the luciferase to oxidize D-luciferin. The nucleotide dATP acts as a substrate for luciferase, thus it is important to use the modified a-S-dATP as the nucleotide for primer extension. Unincorporated nucleotides are eliminated before the new nucleotide is inserted by the apyrase. If the second nucleotide added is not complementary to the template sequence, no PPi is released and the nucleotide is degraded. In the template sequence there is a potential methylation variable position (CpG site) where C (methylated cytosine before bisulfite treatment) or T (unmethylated cytosine before bisulfite treatment) can be incorporated and the respective ratio yields the methylation degree at this CpG position. If 50% of the molecules incorporate a C, we can seen a peak of half of the intensity compared to first A peak corresponding to a single nonpolymorphic nucleotide incorporation. The other half of the molecules is complementary to the T added after the dispensation of the C nucleotide and the degradation of any unincorporated Cs. If the other two nucleotides in the template sequence are identical, these result in a peak of twice the height compared with the first A peak (Tost and Gut, 2007).

Many techniques used to investigate DNA methylation, particularly those that are based on methylation specific PCR (MSP), assume that DNA methylation is homogeneous, making data obtained from heterogeneously methylated regions difficult to interpret; amplicons derived from fully methylated sequences will not form heteroduplex with amplicons derived from fully unmethylated sequences. When methylation is heterogeneous, heteroduplex form because of the presence of molecules that differ only by a few bases. The MS-HRM is capable of analyzing homogeneous methylation in a semiquantitative manner. Heterogeneous methylation is not quantifiable, but is recognizable by its characteristic melting profiles. Samples that show an earlier melt compared to unmethylated control are heteroduplex that have low levels of DNA methylation. Other samples that finish melting shortly after the unmethylated control could have low or moderate levels of DNA methylation that is also heterogeneous. The lowest mean value of methylation of a sample analyzed with PYRO can be explained either by the presence of very few heavily methylated epialleles in a large background of unmethylated alleles or by the presence of epialleles which have only a smaller number of GpG dinucleotides methylated across the entire amplicon. MS-HRM and pyroseqencing provide to complementary information for the assessment of heterogeneous methylation (Candiloro *et al*, 2011).

Materials and Methods

1. Study population

Surgically resected tumor tissues and adjacent normal tissues (at 20 cm of distance) were collected from 59 patients (mean age= 69.7±11.7); staging was assessed after pathological examination of specimens based on TNM classification (Table 3). Not all the healthy tissue are available. For the other samples at the moment there is no available histological material. Blood samples have been also collected from the patients to obtain plasma folate, homocysteine and vitamin B12 values and to analyze polymorphisms in folate metabolism genes. The study was approved by the ethical committee and is sponsored by ITT. The individuals gave their written informed consent. The samples were obtained from the Department of Surgery of University of Pisa (Prof. P. Miccoli). The study includes also the recruitment of 9 patients (mean age= 72.3± 8.8) obtained from the Medical School, Institute for Ageing and Health, University of Newcastle, UK (Prof. J. Mathers) (Table 3).

Table 3: Demographics characteristics of the study population

Patients	Gender	Age	Duke's stage (TNM stage)	Tumor size	Cancer Location *
P2	F	61	C (Stage III)	T3	C
P3	M	42	B (Stage II)	T3	C
P4	M	79	n.a	n.a	n.a
P5	M	73	n.a.	T1	n.a.
P6	F	86	B (Stage II)	T2	C
P7	F	78	n.a	n.a	n.a
P8	M	63	A (Stage I)	T1	C
P9	F	62	B (Stage II)	T3	C

P10	M	68	C (Stage III)	T3	C
P11	M	76	B (Stage II)	T3	C
P12	F	80	C (Stage III)	T3	C
P13	M	71	C (Stage III)	T3	SR
P14	M	58	C (Stage III)	T3	C
P15	F	70	A(Stage 0)	Tis	n.a.
P16^{INK4A}	F	71	A (Stage I)	T2	C
P17	F	47	C (Stage III)	T3	S
P18	M	77	A (Stage I)	T2	C
P19	M	72	C(Stage III)	T3	SR
P20	F	74	D (Stage IV)	T3	C
P21	M	85	B (Stage II)	T3	C
P22	F	79	D (Stage IV)	T3	SR
P23	M	48	B (Stage II)	T3	SR
P24	M	87	B (Stage II)	T3	C
P25	M	81	D (Stage IV)	T3	C
p26	M	58	C (Stage III)	T3	SR
P27	M	77	B (Stage II)	T3	S
P28	F	64	A (Stage I)	T2	SR
P29	F	56	C (Stage III)	T3	R
P30	F	84	Tubulo-villous adenoma	Tubulo-villous adenoma	C
P31	F	82	Polyp	Polyp	C
P32	F	82	B (Stage II)	T3	C
P33	M	78	n.a.	n.a.	n.a.
P34	M	73	C (Stage III)	T2	n.a.
P35	M	76	B (Stage II)	T3	C
P36	F	82	B (Stage II)	T3	C
P37	M	64	n.a.	n.a.	S
P38	M	50	B (Stage II)	T3	SR
P39	M	60	B (Stage II)	T3	C
P40	M	68	B (Stage II)	T3	C
P41	F	62	C (Stage III)	T3	C
P42	M	81	A (Stage I)	T2	S
P43	F	85	D (Stage IV)	T4	C

P44	M	48	A (Stage I)	T2	C
P45	F	87	B (Stage II)	T3	C
P46	M	82	C (Stage III)	T3	SR
P47	M	63	D (Stage IV)	T3	C
P48	M	50	n.a.	n.a.	n.a.
P49	F	50	B (Stage II)	T3	SR
P50	M	57	C (Stage III)	T3	S
P51	M	65	A (Stage I)	T2	C
P52	F	85	D (Stage IV)	T4	C
P53	M	70	A (Stage I)	T2	SR
P54	F	52	D (Stage IV)	T4	C
P55	M	64	B (Stage II)	T3	RS
P56	F	70	C (Stage III)	T3	C
P57	M	82	B (Stage II)	T3	C
P58	M	71	D (Stage IV)	T3	C
P59	F	71	C (Stage III)	T3	C
P60	F	52	C (Stage III)	T3	C
42T (Nc)	M	79	B (Stage II)	T2	R
45T (Nc)	M	61	C (Stage III)	T3	R
54T (Nc)	M	61	C (Stage III)	T3	R
55T (Nc)	M	64	B (Stage II)	T2	SR
57T (Nc)	F	75	C (Stage III)	T3	C
63T (Nc)	M	77	C (Stage III)	T3	C
64T (Nc)	M	70	B (Stage II)	T2	S
65T (Nc)	M	78	B (Stage II)	T2	S
48 T (Nc)	F	86	B (Stage II)	T2	C

(* C: Colon; R: Rectum, S: Sigma SR: Sigma-Rectum).

2. Isolation of human epithelial cells

Where possible, human epithelial cells were isolated from colon-rectal cancer and healthy tissues through immunomagnetic method with microbeads coated with the antibody CD326+, specific for epithelial cells. Briefly the method consist of enzymatic

dissociation of tissue fragments with collagenase-H, filtration with sterile filters and epithelial cells purification by immunomagnetic method (Miltenyi Biotec) (Experiments performed in the Prof. Consolini laboratory).

3. Extraction of genomic DNA

Genomic DNA was extracted using QIAmp DNA blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction.

4. Genotyping

The genotyping for *MTHFR* C677T, *MTHFR* A1298C, *TYMS* 28 bp repeats, *TYMS* 6 bp deletiom, *MTR* A2756G, *MTRR* A66G, *DNMT 3B* –C149T, *DNMT 3B*-G*579*T were performed according to PCR-RFLP methods described in Coppede *et al.*, 2006 and Coppedè *et al.*, 2012. All PCR reactions were done separately in a 25 ul reaction volume containing 1.25 units of Taq DNA polymerase (Invitrogen), 1.5 mM of MgCl2, 1x PCR buffer (Invitrogen), 10 pmol of each primers and 30 ng of genomic DNA. All PCR reactions were set with an initial denaturation of 5 min at 95° C and subsequently denatured for 30 s at 95°C, with annealing for 30 s at the appropriate temperature (see table 4) and extension for 30 s at 72°C. Thirty five cycles were used to amplify the PCR products to the expected sizes; the amplicons were subsequently digested using appropriate restriction enzymes (Fermentas). Figure 7 shows an image regarding the electrophoresis gel done to discriminate the different genotypes of *MTHFR C677T* (the images relative to the other polymorphisms are not shown).

Table 4: Primer sequences and annealing temperature used in polymorphisms analyses.

Table 4: Primer sequences and annealing temperature used in polymorphisms analyses.

SNP	Primers (5'-3')	Annealing temperature
MTHFR C677T	F-TGAAGGAGAAGGTGTCTGCGGGA R-AGGACGGTGCGGTGAGAGTG	62°C
MTHFR A1298C	F-CTTTGGGGAGCTGAAGGACTACTA R-CACTTTGTGACCATTCCGGTTTG	60°C
MTR A2756G	F-TGT TCCCAGCTGTTAGATGAAAATC R-GAT CCAAAGCCTTTTACACTCCTC	60°C
MTRR A66G	F-GCAAAGGCCATCGCAGAAGACAT R-5'TGGTGGTATTAGTGTCCTTTTG	56°C
TYMS 28 bp repeats	F-GTGGCTCCTGCGTTTCCCCC R-GGCTCCGAGCCGGCCACAGGCATGGCGCGG	63°C
TYMS 6 bp del	F-CAAATCTGAGGGAGCTGAGT R-CAGATAAGTGGCAGTACAGA	58°C
DNMT3B -149C>T	F-TGCTGGACAGGCAGAGCAG R-GGTAGCCGGGAACTCCACGGG	67°C
DNMT3B -579G>T	F-GAGGTCTCATTATGCCTAGG R-GGGAGCTCACCTTCTAGAAA	56°C

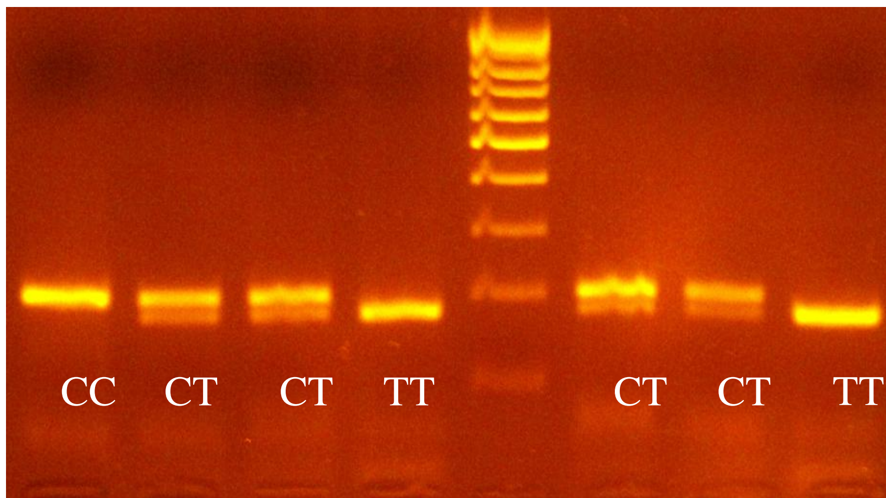


Figure 7: *MTHFR* C677T genotypes: Digestion produced 175 e 23-bp in the presence of 677T allele, a 198bp fragment in the presence of 677C allele.

5. Methylation analysis

The extracted DNA was quantified using a Nano Drop ND 2000c spectrophotometer (NanoDrop Thermo scientific, Wilmington, DE). 250 ng of DNA from each subject have been treated with sodium bisulfite using the "EpiTect® Bisulfite Kit" (Qiagen, Milan, Italy) according to the manufacturer's protocol. Sodium bisulfite treatment

converts all unmethylated cytosines into uracil, whilst methylated cytosines are left unchanged. After sodium bisulfite treatment MS-HRM analysis was performed as described as follows. In this work we compare the analytical performances and the predictive values of two technique (MS-HRM and Pyrosequencing). Protocols for Methylation Sensitive High Resolution Melting (MS-HRM) have been developed in our laboratory (Prof. Lucia Migliore) by modifying literature methods (Wojdacz and Dobrovic, 2007; Huang *et al*, 2010). The Pyrosequencing was performed in the laboratory of Prof. John Mathers (Newcastle University).

Standard curves with known methylation ratios were included in each assay and were used to deduce the methylation ratio of each tumor and normal sample. The HRM standard melting curves were derived from six samples with the following ratios of methylation: 0%, 12,5%, 25%, 50%, 75%, 100%. Fully methylated and unmethylated bisulfite converted DNA (Qiagen) were mixed to obtain the different percentages of methylation.

5.1 MS-HRM protocols

MS-HRM for *APC:* PCR amplification and HRM were performed on CFX 96™ Real-Time (BioRad). The sequences of the primers for *APC* are as follows: forward-CGGGGTTTTGTGTTTTATTG and reverse - TCCAACGAATTACACAACTAC. PCR was performed in a 25 ul volume containing: 12,5 ul of master mix (Qiagen) 10 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 56 °C for 45 s, and 72°C for 45 s; followed by an HRM step of 95°C for 10 s and 50°C for 1 min, 65° C for 15 s, and continuous acquisition to 95°C at 1 acquisition per 0.2°C. The PCR product was of 71 bp (4 CpG sites analyzed). Figure 8 shows the

melting curves regarding the standards and one sample in duplicate (50-75% of methylation)

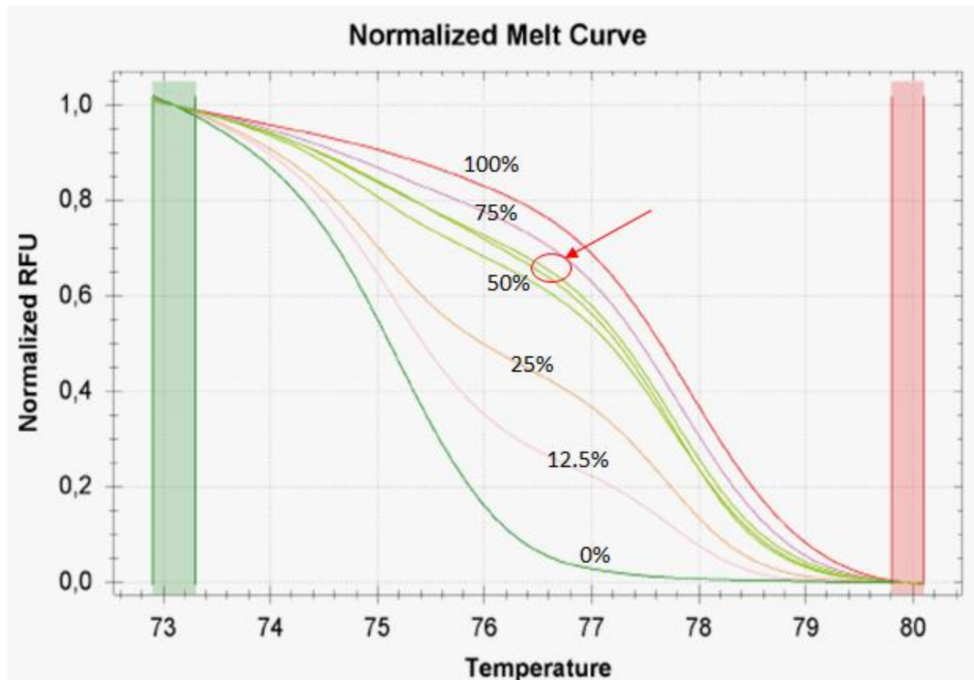


Figure 8: melting curves of *APC* gene: the standards and a sample in duplicate (highlighted)

MS-HRM for *CDKN2A*: PCR amplification and HRM were performed on CFX 96™ Real-Time (BioRad). The sequences of the primers for *CDKN2A (p16^{Ink4A})* are as follows: forward- CGGAGGAAGAAAGAGGAGGGGT and reverse CGCTACCTACTCTCCCCCTCT. PCR was performed in a 25 ul volume containing: 12,5 ul of master mix (Qiagen) 10 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 45 s; followed by an HRM step of 95°C for 10 s and 50°C for 1 min, 68° C for 15 s, and continuous acquisition to 90°C at 1 acquisition per 0.2°C. The PCR product was of 93 bp (7 CpG sites analyzed). Figure 9 shows the melting curves regarding the standards and two samples in duplicate with 12.5-25% of methylation (red arrow) and 0% of methylation (yellow arrow).

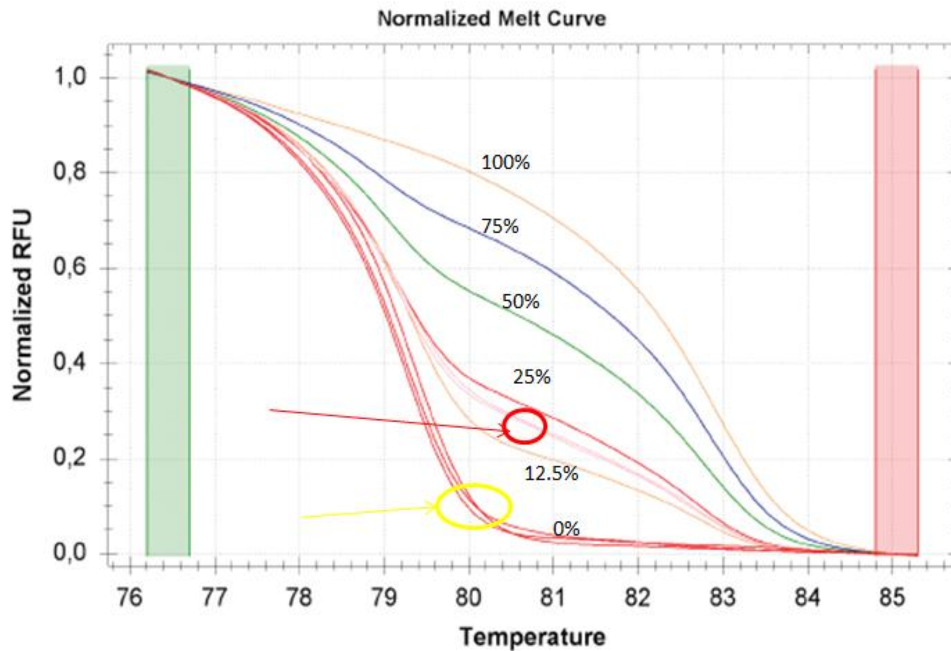


Figure 9: melting curves of *CDKN2A* gene: the standards and two samples in duplicate (highlighted)

MS-HRM for *MGMT:* PCR amplification and HRM were performed on CFX 96™ Real-Time (BioRad). The sequences of the primers for *MGMT* are as follows: forward-GCGTTTCGGATATGTTGGGATAAGT and reverse- AACGACCCAAACACTCACCAAA. PCR was performed in a 25 ul volume containing: 12,5 ul of master mix (Qiagen) 10 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 30 s; followed by an HRM step of 95°C for 10 s and 50°C for 1 min, 68° C for 15 s, and continuous acquisition to 90°C at 1 acquisition per 0.2°C. The PCR product was of 110 bp (12 CpG sites analyzed). Figure 10 shows the melting curves regarding the standards and two samples in duplicate with 0-12.5% of methylation.

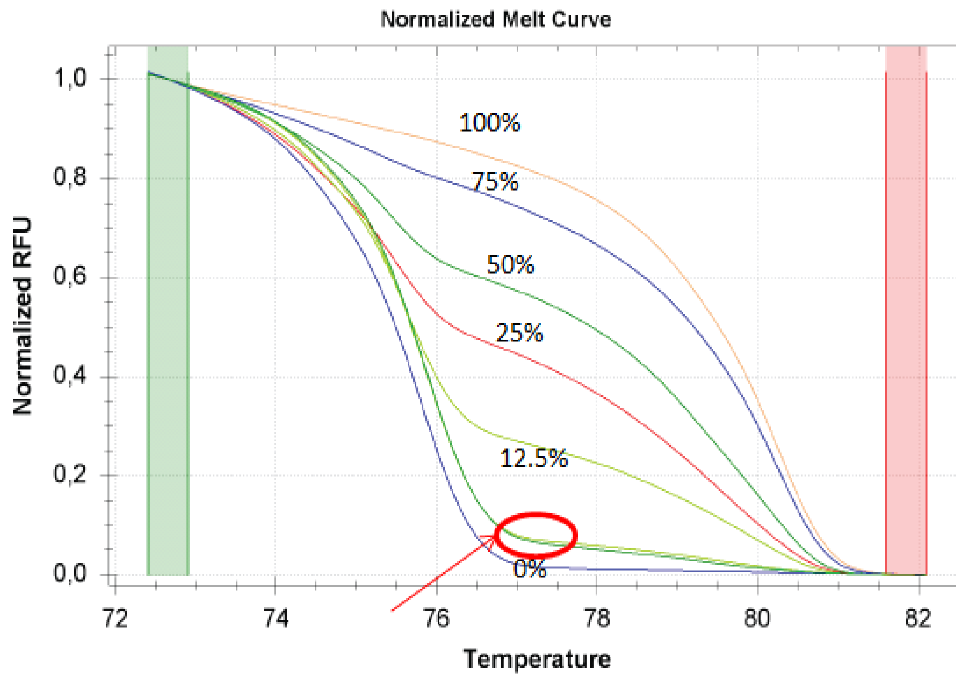


Figure 10: melting curves of *MGMT* gene: the standards and two samples in duplicate (highlighted)

MS-HRM for *hMLH1*: PCR amplification and HRM were performed on CFX 96™ Real-Time (BioRad). The sequences of the primers for *hMLH1* are as follows: forward-AGTTTTTAAAAAC/TGAATTAATAGGAAGAG and reverse-ACTACCCGCTACCTAAAAAAATATAC. PCR was performed in a 25 ul volume containing: 12,5ul of master mix (Qiagen), 15 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 15 s, 56°C for 15s, and 72°C for 15s; followed by an HRM step of 95°C for 30 s and 50°C for 1 min, 65° C for 15 s, and continuous acquisition to 95°C at 1 acquisition per 0.2°C. The PCR product was of 81 bp (5 CpG sites analyzed). Figure 11 shows the melting curves regarding the standards and two samples in duplicate with 0 % of methylation.

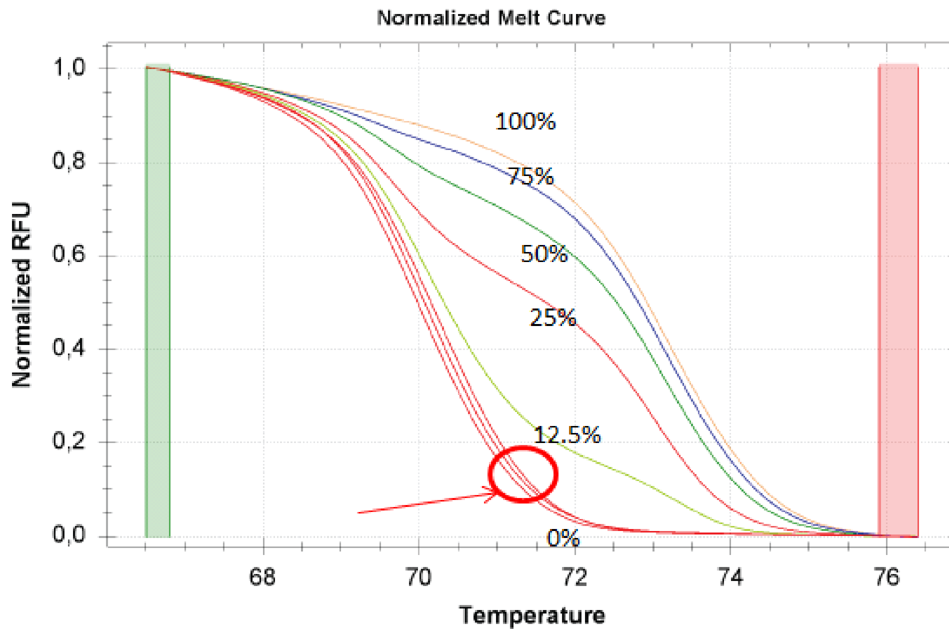


Figure 11: melting curves of *h MLH1* gene: the standards and a sample in duplicate (highlighted)

MS-HRM for *hMLH1:* It is also performed a MS-HRM with primers used in Pyrosequencing (. PCR amplification and HRM were performed on CFX 96™ Real-Time (BioRad). The sequences of the primers for *hMLH1* are as follows: forward-GGTTATAAGAGTAGGGTTAA and reverse- ATACCAATCAAATTTCTC. PCR was performed in a 25 ul volume containing: 12,5ul of master mix (Qiagen), 15 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 48°C for 15s, and 72°C for 30s; followed by an HRM step of 95°C for 10 s and 50°C for 1 min, 68° C for 15 s, and continuous acquisition to 85°C at 1 acquisition per 0.2°C. PCR product was of 307 bp (18 CpG sites analyzed). Figure 12 shows the melting curves regarding the standards and two samples in duplicate with 50-75% of methylation

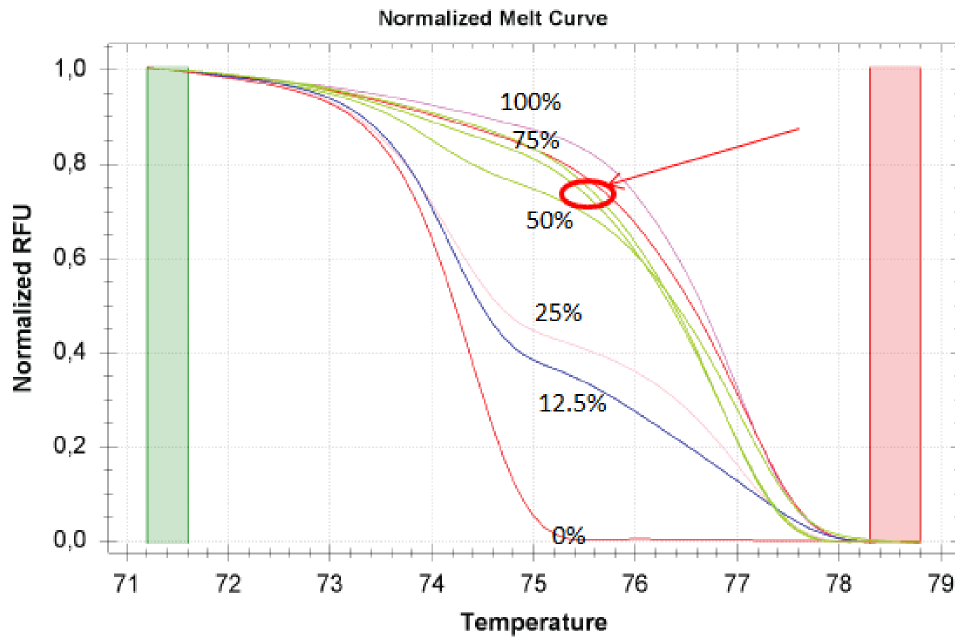


Figure 12 melting curves of *h MLH1* gene (with Pyrosequencing primer): the standards and a sample in duplicate (highlighted)

MS-HRM for *RASSF1A:* PCR amplification and HRM were performed by the CFX 96™ Real-Time (BioRad).The sequences of the primers for *hRASSF1A* are as follows: forward- TCGGGTTTTATAGTTTTTGTATTTAGGTTTT and reverse-CCTCCCCCAAAATCCAAACTAA. PCR was performed in a 25 ul volume containing: 12,5 ul of master mix (Qiagen) 10 pmol of each primer and 50 ng bisulfate treated DNA template. Each reaction was performed in duplicate. The cycling conditions were: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s; followed by an HRM step of 95°C for 10 s and 50°C for 1 min, 65° C for 15 s, and continuous acquisition to 95°C at 1 acquisition per 0.2°C. The PCR product was of 87 bp (7CpG sites analyzed). Figure 13 shows the melting curves regarding the standards and two samples in duplicate with 0% of methylation.

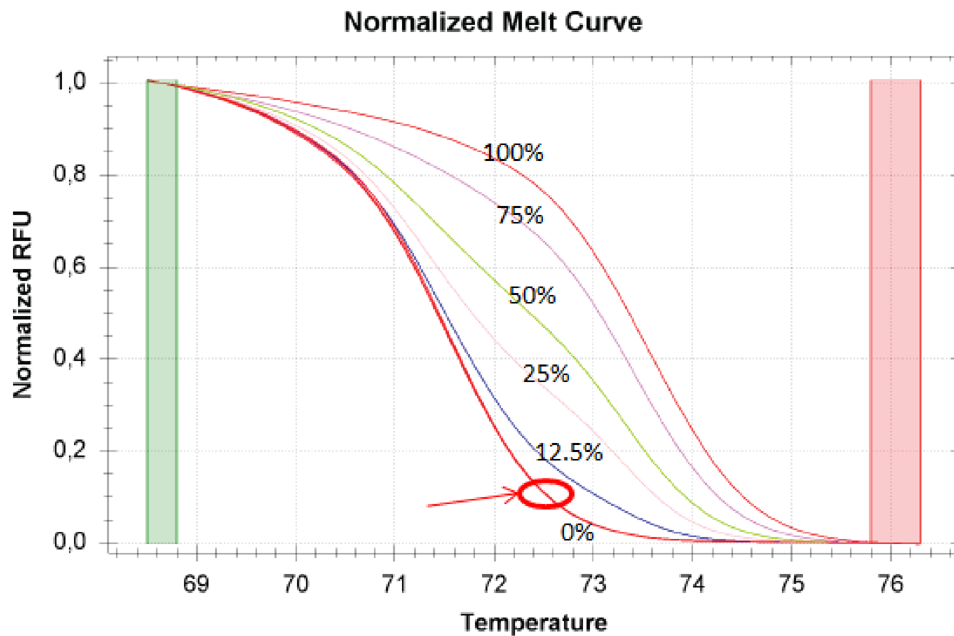


Figure 13: melting curves of *RASSF1A* gene: the standards and a sample in duplicate (highlighted)

5.2 Pyrosequencing protocols

Genomatix software (www.genomatix.de) Gene2Promoter allows to look for a promoter regions within sequenced genomes of various organisms. In parallel in Genomotix it is possible to see transcription factor binding sites.

PSQ software was used to design the primers. Bisulfite modified (BM) 0% and 100% DNA should be used in a pre-PCR dilution series to produce 0, 25, 50, 75, 100% BM DNA. These dilutions should be used for subsequent Pyrosequencing PCR. Usually the standard conditions for the PyroPCR are: 12,5 of Taq Mastermix (Qiagen), 10pmol of each primers and 250 ng of BM DNA in a total volume of 25 μL. The PyroPCR temperature profile was the follow: 95°C for 15min, 94°C for 15 s, X°C for 30 s, 72°C for 30 s (Repeat steps 2, 3, 4 x50 times) and 72°C for 10 min. After PyroPCR, the DNA concentration varies due to the reaction condition, but it will usually fall in the region 15-30 ng/μL. Figures 14 and 15 show the *APC* and *CDKN2A* Pyrosequencing validation with pre-PCR standard dilution. It is reported only the first CpG site of the analyzed

region. For the other two genes (*MGMT* and *hMLH1*) it was utilized the standardized protocol used in John Mathers laboratory.

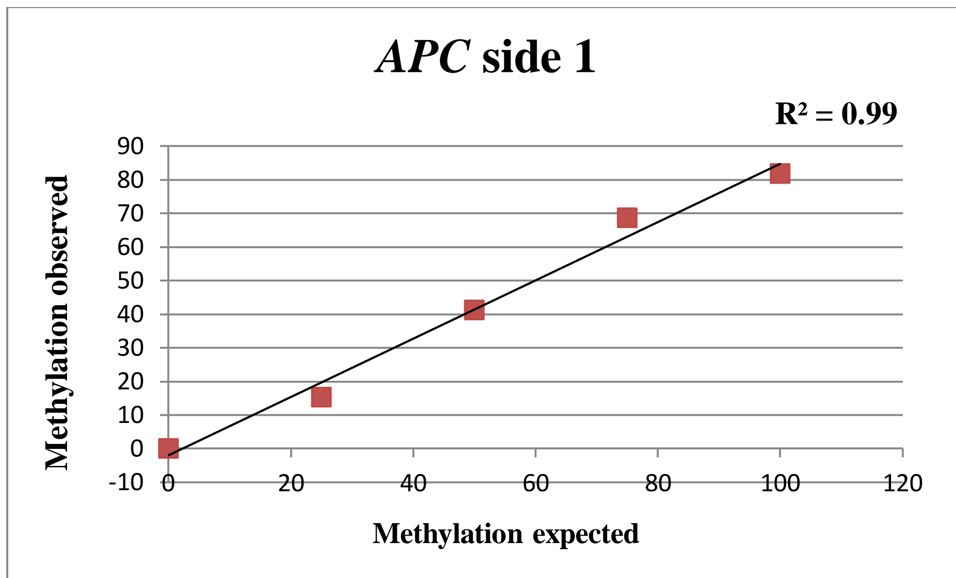


Figure 14: *APC* Pyrosequencing validation

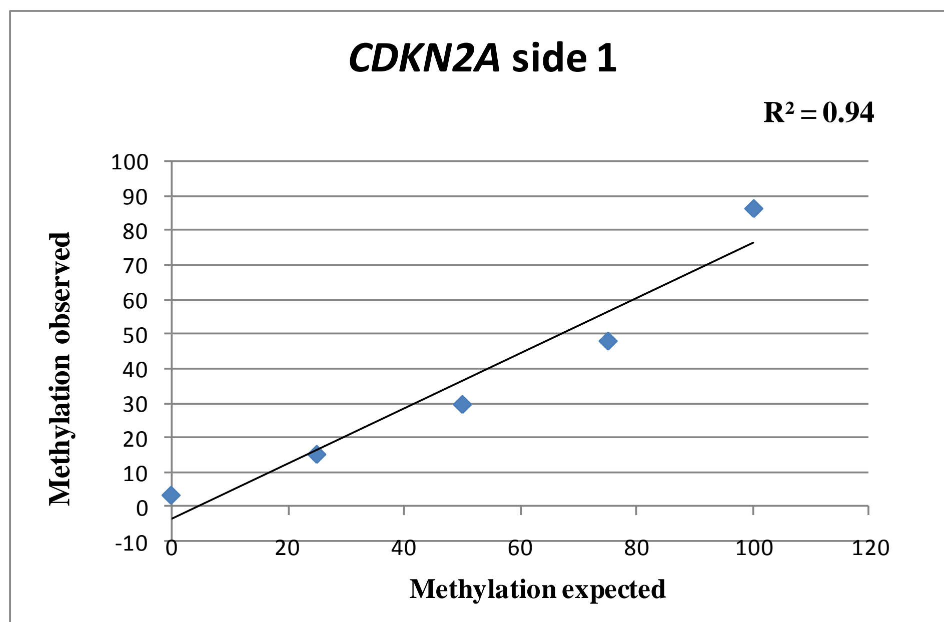


Figure 15: *CDKN2A* Pyrosequencing validation

Biotinylated PCR products are immobilised on streptavidin-coated beads. Parallel immobilisation of several samples can be performed in a sealable 96 well PCR plate. It is important: 1)Allow all solutions to reach room temperature before starting; 2) Turn on heat block to 80°C; 3) Shake bottle of streptavidin beads to obtain a homogenous solution.

The Binding buffer solution is constituted by 2µl beads, 38µl binding buffer and 30µl H20. The passages are: transfer 70µl of this solution into a PCR plate (shaking after every few wells to prevent beads settling). Thus add 10µl of PyroPCR product to binding buffer solution in PCR plate (NB: when the PCR product on gel shows very strong band, you could use only 5 µl). Subsequently:

1. Seal plate and shake for 10mins.
2. Whilst the plate is shaking prepare the Pyrosequencing plate by making the annealing solution. Make a master mix with the following volumes per sample: 11.5µl of annealing buffer and 0.5µl (5pmol/µl) of sequencing primer. Add 12µl of this to each corresponding well on a pyro plate.

For the strand separation, fill four square plastic troughs with appropriate solutions up to the line of troughs:

	probe block dH20
2.Denaturing buffer (NaOH)	4.dH20
1.Ethanol	3.Wash buffer
PCR plate	Pyro plate

1. Switch on vacuum pump and prime the probes by sucking through dH20 for a few sec.

2. Remove plate from shaker and immediately apply vacuum sample prep tool (head). Make sure all the sample has been taken up from the plate.

3. Follow the pattern 1→2→3, so:

4. Place vacuum tool in ethanol trough – once liquid flows through tool count 5 sec and remove, leaf to dry a bit. Repeat this for the denaturing and then wash buffer.

5. Turn off vacuum, place tool into Pyrosequencing plate to release the single stranded PCR product into the annealing solution – rock or shake for several sec

6. Place the plate on to the pre-heated heat block (80°C) and leave for 2min. Clean the vacuum tool by sucking H20 through for a few seconds.

7. Remove plate and leave to cool for a few minutes, allowing the primer to anneal to PCR product.

8. The plate is now ready for the Pyrosequencing.

Thus it is necessary filling the reagent cartridge and place it with label facing you in the Pyrosequencer. Each tip should be labeled with the reagent it is used for, i.e. regent tips labeled E or S for enzyme and substrate and nucleotide tips labeled A, C, G or T. The enzyme tip should be filled with the appropriate amount of enzyme for assay and placed in the top left. The substrate tip should be filled with the appropriate amount of substrate for assay and placed in the top right. Nucleotides tips should be filled with care – sometimes air bubbles form in bottom which can interfere with dispensation – to remove flick tip. In table 5 are shown the different conditions (primers and annealing temperature) regards to genes analyzed by Pyrosequencing. Figures 16, 17 and 18

represent the images relatives to the CpG sites of the three promoter gene regions analyzed by Pyrosequencing.

Table 5: Pyrosequencing conditions and analyzed sequences

Genes	Primer Pyrosequencing	Annealing temperature	CpG sites/amplicon lenght
APC	F-TATTAATTTTTTTGTTTGTTGGGGA R-AACTACACCAATACAACCACATATC Sequencing primer: GGGGTTTTGTGTTTTATTG	55 C	C/TGGAGTGC/TGGGTC/TGGGA AGC/TGGAGAGAGAAGTAGTT GTGTAATTC/TGTTGGATGC/TG GATTAGGGC/TGT
CDKN2A	F-AGAGGATTTGAGGGATAG R-AATTCCCCTACAAACTTC Sequencing primer: GGGTTGGTTGGTTATTA	50 C	GAGGGTGGGGC/TGGATC/TGC /TGTGC/TGTTC/TGGC/TGGTTG C/TGGAGAGGGGGAGAGTAGG TAGC/TGGGC/TGGC/TG
hMLH1	F-GGTTATAAGAGTAGGGTTAA R-ATACCAATCAAATTTCTC Sequencing primer: TGTTTTTATTGGTTGGATAT	45 C	TTC/TGTATTTTTC/TGAGTTTTT AAAAAC/TGAATTAATAGGAA GAGC/TGGATAGC/TGATTTTTA AC/TGC/TGTAAGC/TGTA
*MGMT**	F-AGTTTTTTTGGTGGATATA R-TACCTTTTCCTATCACAA Sequencing primer: TTTAGGAGGGGAGAGAT	47 C	TC/TGC/TGTTTC/TGGGTTTAG C/TGTAGTC/TGTTTC/TGAGTA GGATC/TGGGATTTTTATTAAG

(* CpG island B)

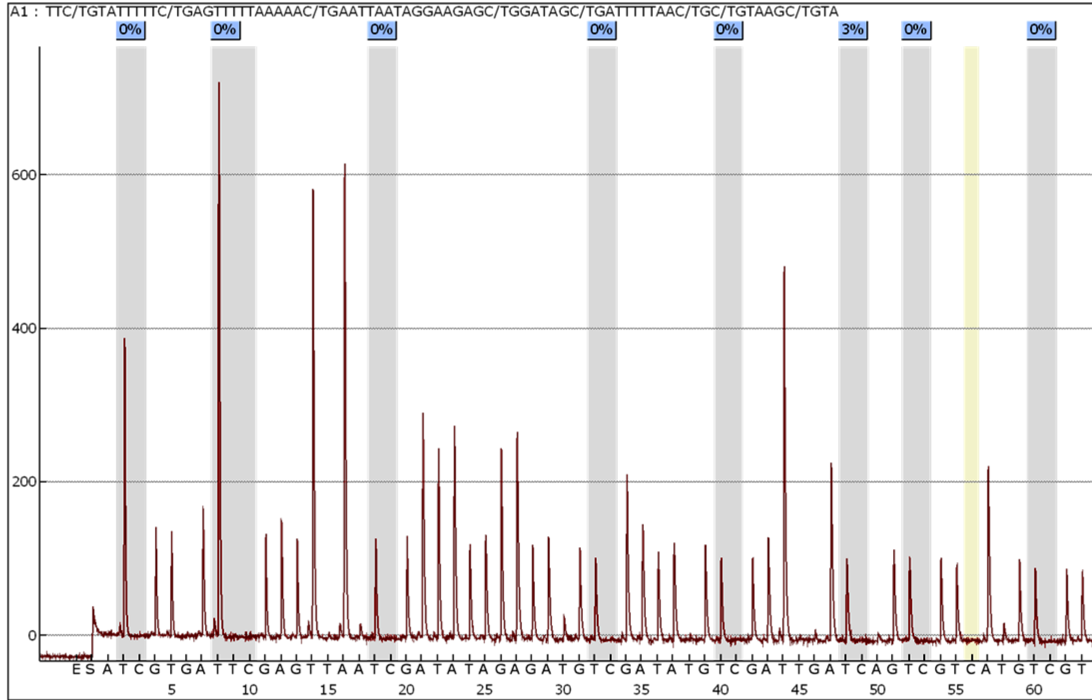


Figure 16: *hMLH1* Pyrosequencing- 8 CpG sites analyzed in the CpG Island of *hMLH1* gene. The y axis represents the signal intensity, while the x axis shows the dispensation order. The azure color indicate the % of methylation at each CpG site.

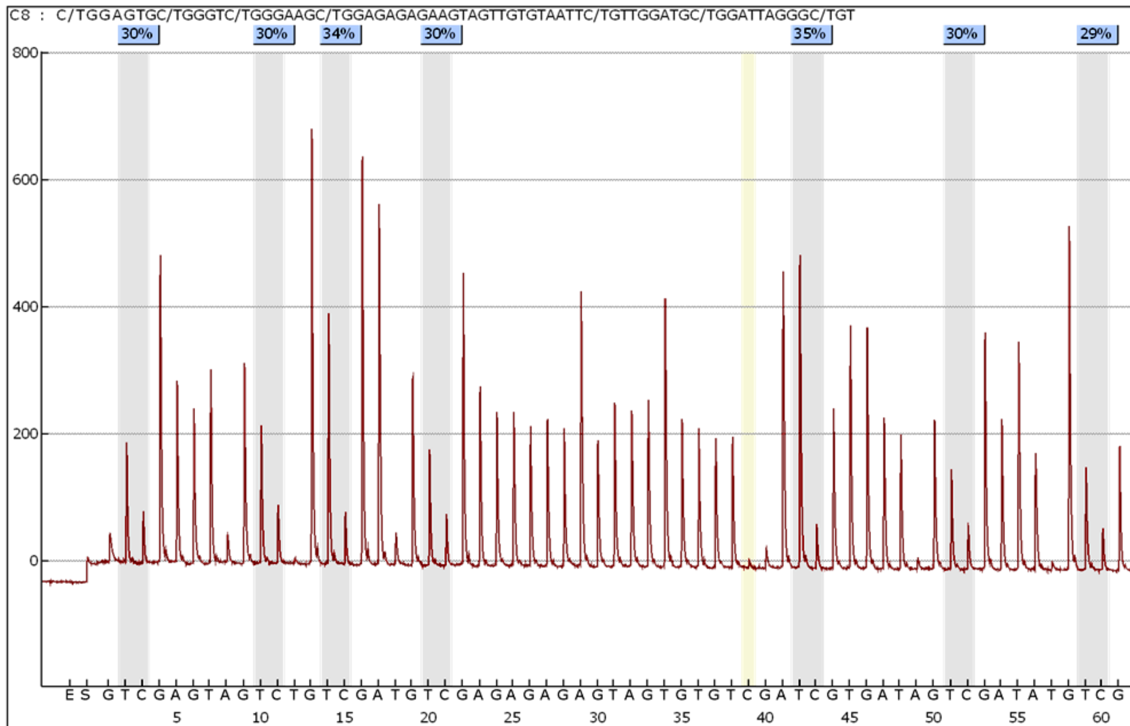


Figure 17: *APC* Pyrosequencing: 7 CpG sites analyzed in the CpG Island of the *APC* gene. The y axis represents the signal intensity, while the x axis shows the dispensation order. The azure color indicate the % of methylation at each CpG site.

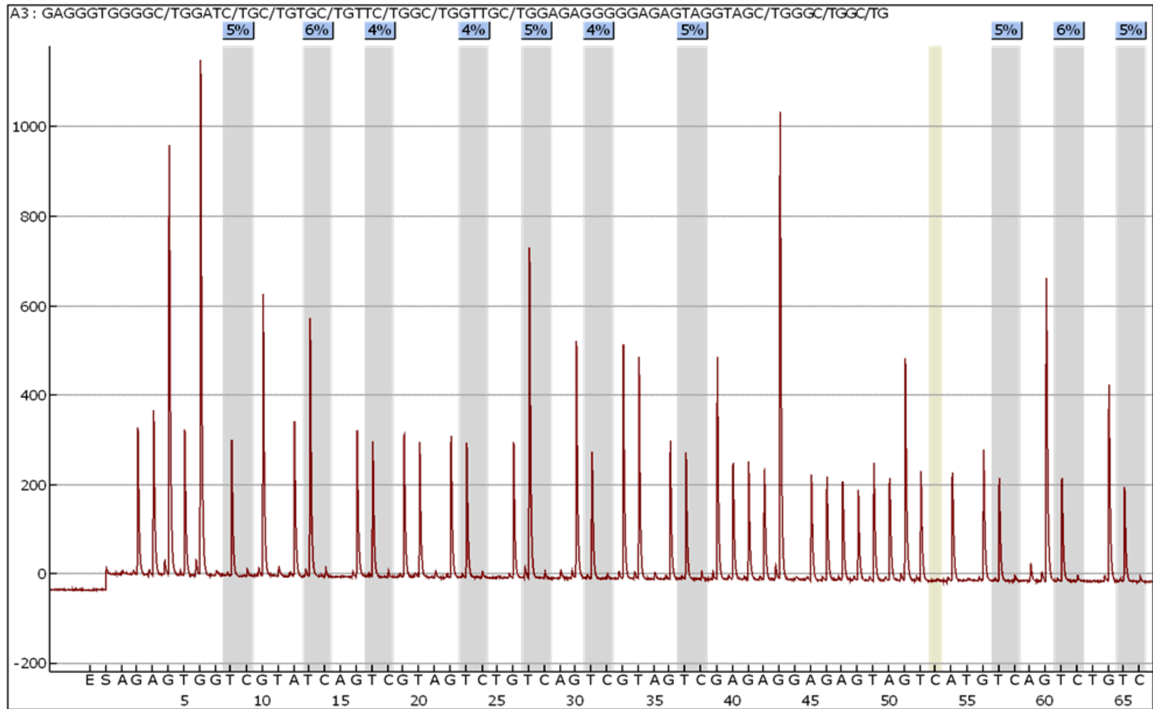
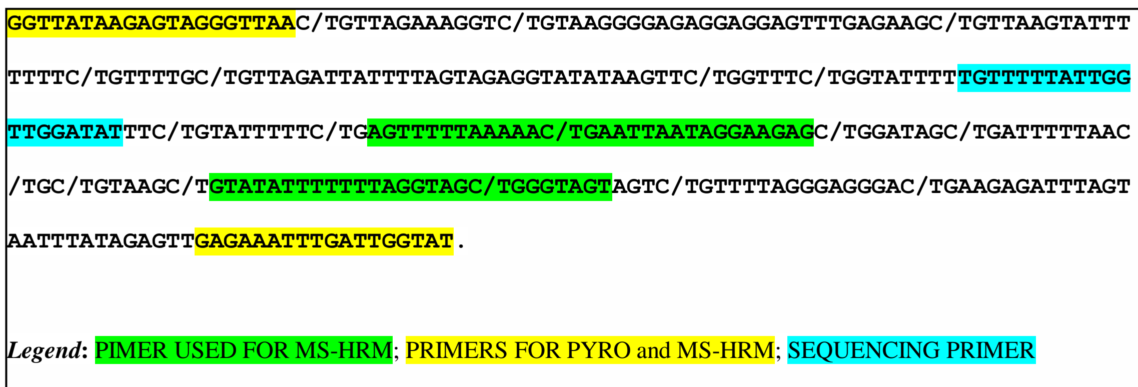


Figure 18: *CDKN2A* Pyrosequencing: 10 CpG sites analyzed in the CpG Island of *the CDKN2A* gene. The y axis represents the signal intensity, while the x axis shows the dispensation order. The azure color indicate the % of methylation at each CpG site.

Note 1: For *hMLH1* methylation analysis it was used two different couples of primer for MS-HRM: one set of primer (in green) and another set of primer (blue) also used in Pyrosequencing. The azure primer is the sequencing primer for *hMLH1*; thus the sequence analyzed in Pyrosequencing was least then MS-HRM sequence. Figure 19 shows the whole analyzed sequence.



GGTTATAAGAGTAGGGTTAAC/TGTTAGAAAGGTC/TGTAAGGGGAGAGGAGGAGTTTGAGAAGC/TGTTAAGTATTT TTTTC/TGTTTTGC/TGTTAGATTATTTTAGTAGAGGTATATAAGTTC/TGGTTTC/TGGTATTTTTGTTTTTATTGG TTGGATATTTC/TGTATTTTTC/TGAGTTTTTAAAAAC/TGAATTAATAGGAAGAGC/TGGATAGC/TGATTTTTAAC /TGC/TGTAAGC/TGTATATTTTTTTAGGTAGC/TGGGTAGTAGTC/TGTTTTAGGGAGGGAC/TGAAGAGATTTAGT AATTTATAGAGTTGAGAAATTTGATTGGTAT.

Legend: PIMER USED FOR MS-HRM; PRIMERS FOR PYRO and MS-HRM; SEQUENCING PRIMER

Figure 19: *hMLH1* sequence analyzed with both Pyrosequencing and MS-HRM.

Note2: For *MGMT* methylation analysis we studied two different CpG island (A and B) regards to Pyrosequencing and MS-HRM and thus data from the two techniques are not comparable.

6. Statistical analysis

A data interpolation was performed to compare MS-HRM and Pyrosequencing methods: a curve according to the method of Polynomial interpolation was obtained. For this purpose the MatLab program and its function interpolating "polyfit" was used. The result coincides with that obtainable with the method of Lagrange interpolation and allows, starting from a note fluorescence, to find the respective percentage of methylation instead of a range of interest. Thus Student's t-test was used to compare MS-HRM and Pyrosequencing values. ANCOVA was used to compare CpG sites methylation in ephitelial cells (CD326+) respects to the whole tumor tissue, using sample ID as covariate. ANCOVA was also used to correlate the *APC, CDKN2A, MGMT, hMLH1, RASSF1A* promoter methylation (obtained by means of MS-HRM) with clinic pathological characteristics. Finally ANCOVA was used to correlate CpG sites methylation of the four genes (*APC, CDKN2A MGMT and hMLH1*), analyzed by Pyrosequencing, with the *MTHFR* C677T and *DNMT3B* C-149T polymorphisms, including age, cancer location, gender, simple ID, tumor size and stage as covariates. Analyses were performed with the STATGRAPHICS 5.1 Plus software package for Windows.

Aim of the study

This thesis is a part of an ongoing project entitled "Correlation among Genetic, Epigenetic and Environmental factors in Colorectal Carcinoma"; it aims to study the relationship among diet factors,, focusing on folate, homocysteine, and vitamin B12, and polymorphisms of one carbon metabolism pathway genes, as well as methylation levels of CRC critical genes (*APC*, *CDKN2A, hMLH1, MGMT* and *RASSF1*A). For the study the recruitment of almost 200 CRC patients within three years is planned. The study will end on January 2013.

This thesis is focused on a cohort of CRC patients recruited until end of February 2012. Moreover, in the frame of a collaboration with Prof. John Mathers (Institute for Ageing and Health, University of Newcastle, UK) a group of 9 CRC patients recruited in UK was also included for some comparisons.

The study comprises the following items:

1. Validation of the MS-HRM protocol, used for the methylation analysis, by comparing it with widely employed technique: Pyrosequencing.
2. Evaluation of the influence of an immunomagnetic method with microbeads coated with the antibody CD326+, specific for epithelial cells, to evaluate if cancer epithelial (CD326+) cells from the surgically resected CRC tissue could give more accurate results in DNA methylation levels detection with respect to the whole CRC tissue.
3. Detection of methylation levels (performed by MS-HRM) in promoters of of *APC*, *CDKN2A, hMLH1, MGMT* and *RASSF1*A genes in CRC and healthy adjacent tissue specimens.

4. Analysis of the correlation among the methylation status of the chosen genes and the clinical-pathological features of the patients.
5. Analysis of the correlation among *MTHFR* C677T, *DNMT3B* C-149T polymorphisms and the methylation levels of *APC, CDKN2A, hMLH1* and *MGMT* gene promoters.

Results

1. Comparison between tumor and healthy tissue in CRC patients

The sensitivity of MS-HRM analysis was tested by using diluitions of fully methylated DNA into unmethylated DNA. The HRM standard melting curves were derived from six samples with the following ratios of methylated DNA: 0, 12.5, 25, 50, 75, 100% of methylation. The normalized melting profiles of the amplicon from the same template were consistent between replicates and between different runs. From each CRC patient either a sample of tumor tissue, either a sample of healthy tissue, located near the cancerous lesion (about 20 cm distance) was collected. Tables 6, 7, 8, 9, 10 show the methylation data obtained in CRC tissues and adjacent healthy colon tissues (not always available for all the patients) obtained by means of MS-HRM for each of the five screened genes (*APC*, *CDKN2A*, *MGMT*, *hMLH1*, *RASSF1A*). It is possible to see that for each of them, methylation is more frequent in CRC tissue samples. Only in case of *APC* gene a consistent increase of methylation in the range 0-12.5% was found also in adiacent healthy tissue.

Table 6: MS-HRM in *APC* gene: percent of methylation in CRC and healthy adjacent tissue

APC **gene promoter methylation**	**CRC tissue Total n° = 62**	**Adjacent healthy tissue Total n° = 46**
0%	26 (42 %)	28 (61%)
0-12.5%	13 (21%)	18 (39%)
12.5-25%	8 (13 %)	0 (0%)
25-50%	8 (13%)	0 (0%)
50-75%	5 (8%)	0 (0%)
75-100%	2 (3%)	0 (0%)

Table 7: MS-HRM in *CDKN2A* gene: percent of methylation in CRC and healthy adjacent tissue

CDKN2A **gene promoter methylation**	**CRC tissue Total n° = 62**	**Adjacent healthy tissue Total n° = 48**
0%	46 (74%)	47 (98%)
0-12.5%	9 (14 %)	1 (2%)
12.5-25%	1 (2%)	0 (0%)
25-50%	1 (2%)	0 (0%)
50-75%	5 (8%)	0 (0%)
75-100%	0 (0%)	0 (0%)

Table 8: MS-HRM in *hMLH1* gene: percent of methylation in CRC and healthy adjacent tissue

hMLH1 **gene promoter methylation**	**CRC tissue Total n° = 62**	**Adjacent healthy tissue Total n° = 45**
0%	53 (85%)	43 (96%)
0-12.5%	3 (5%)	2 (4%)
12.5-25%	1 (2%)	(0%)
25-50%	1 (2%)	(0%)
50-75%	2 (3%)	(0%)
75-100%	2 (3%)	(0%)

Table 9: MS-HRM in *RASSF1A* gene: percent of methylation in CRC and healthy adjacent tissue

***RASSF1A* gene promoter methylation**	**CRC tissue Total n° = 60**	**Adjacent healthy tissue Total n° = 46**
0%	53 (89%)	46 (100%)
0-12.5%	0 (0%)	0 (0%)
12.5-25%	2 (3%)	0 (0%)
25-50%	2 (3%)	0 (0%)
50-75%	3 (5%)	0 (0%)
75-100%	0 (0%)	0 (0%)

Table 10: MS-HRM in *MGMT* gene: percent of methylation in CRC and healthy adjacent tissue

***MGMT* gene promoter methylation** *	**CRC tissue Total n° = 59**	**Adjacent healthy tissue Total n° = 45**
0%	38 (65%)	39 (87%)
0-12.5%	5 (8%)	6 (13%)
12.5-25%	4 (7%)	0 (0%)
25-50%	5 (8%)	0 (0%)
50-75%	3 (5%)	0 (0%)
75-100%	4 (7%)	0 (0%)

*CpG Island A

2. Correlation between methylation and clinical-pathological features

For each of the studied genes we also analysed correlation between promoter TNM stage methylation by ANCOVA analysis of regression analysis multifactorial analysis of variance, including age, gender, tumor, size, tumor location as covariates. Figures 20, 21, 22, 23, 24 show the correlation between methylation of analyzed genes and staging of cancers, using the other variables as covariates. No statistical association was found in any case. Statistically significant associations were found instead between *MGMT* promoter methylation (Figure 24) and increasing age, and between *hMLH1* promoter methylation and increasing age (Figure 25).

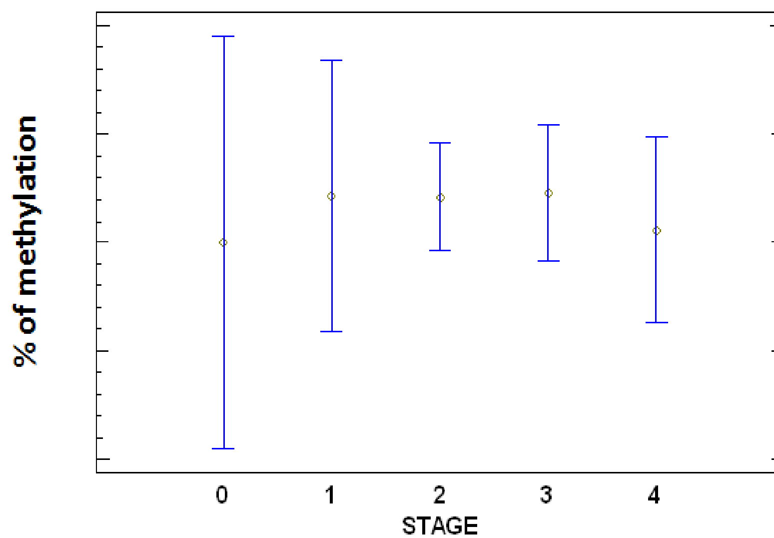


Figure 20: Correlation between *APC* promoter methylation and TNM stage

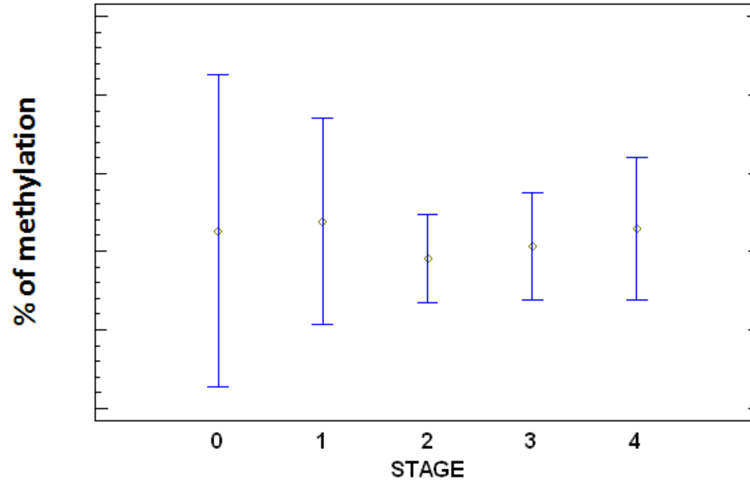


Figure 21: Correlation between *MGMT* promoter (CpG island A) methylation and TNM stage

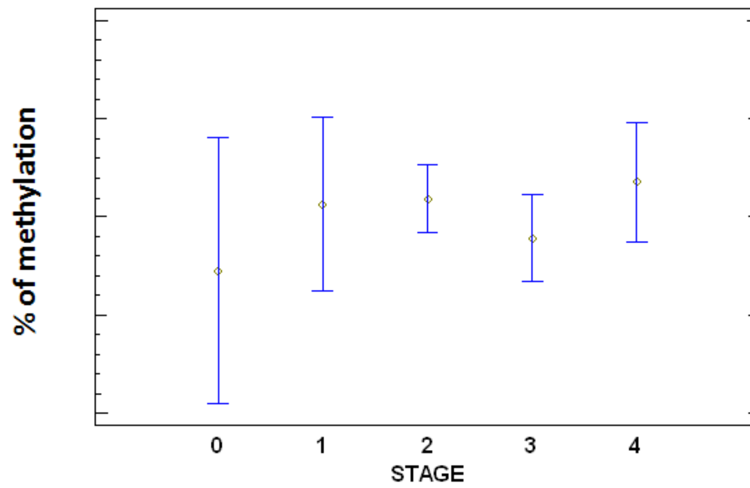


Figure 22: Correlation between *hMLH1* promoter methylation and TNM stage

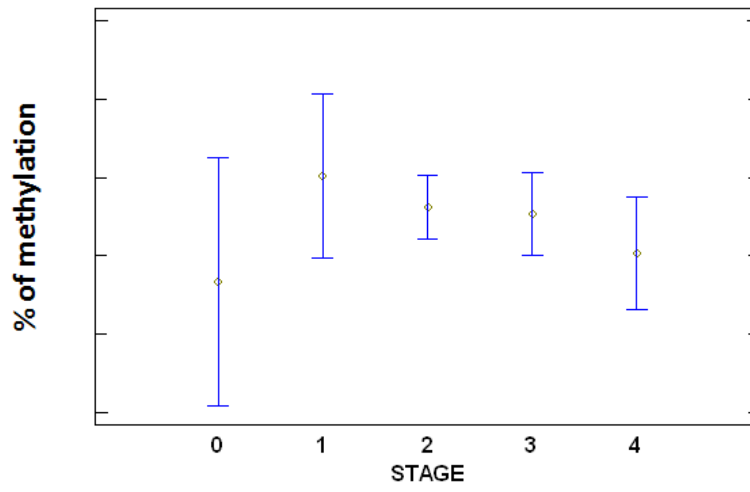


Figure 23: Correlation between *CDKNN2A* promoter methylation and TNM stage

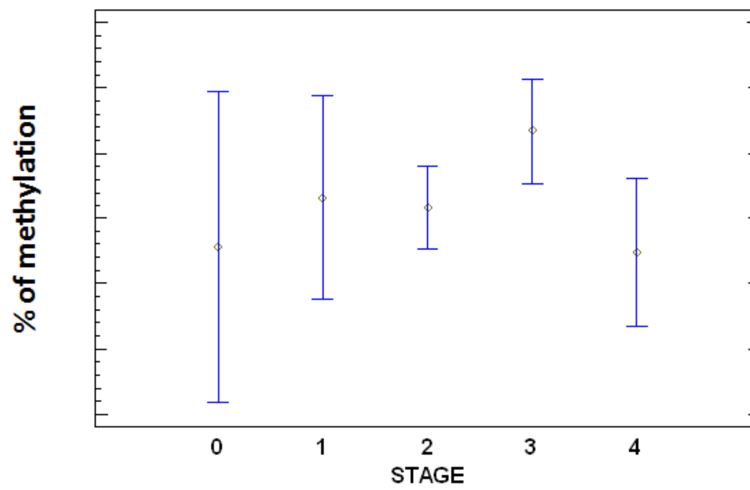


Figure 24: Correlation between *RASSF1A* promoter methylation and TNM stage

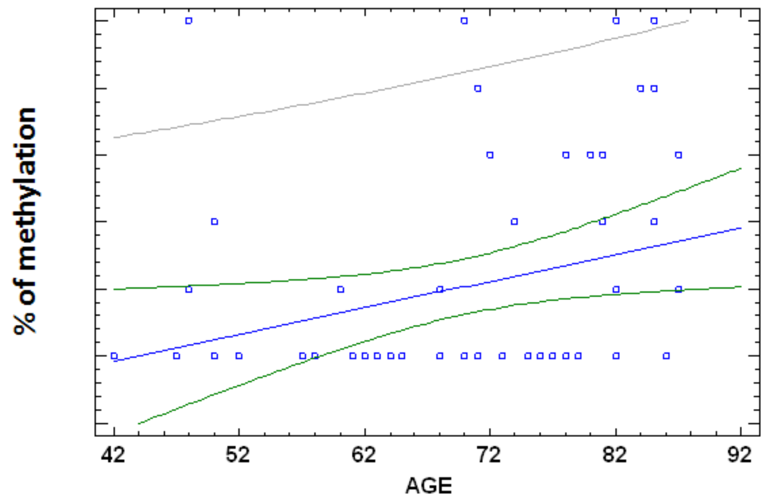


Figure 25: Correlation between *MGMT* promoter methylation (HRM results) and age: r =0.28, *P*=0.03

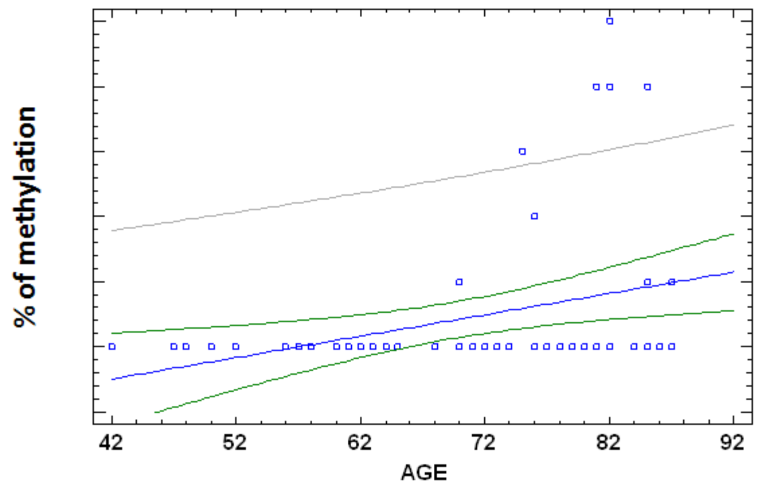


Figure 26: Correlation between *hMLH1* promoter methylation (HRM results) and age: r =0.35 , P=0.007

3. Comparison between MS-HRM and Pyrosequencing techniques

Experiments were performed to compare two different techniques (MS_HRM and Pyrosequencing) used to investigate DNA methylation levels. Figures 27, 28, 29, 30, 31, 32 show the methylation profiles obtained for each patient with the two methods (analysis of three genes: *hMLH1*, *APC* and *CDKN2A*). In each case we obtained specular profiles of the methylation and no statistically significant difference in mean values, indicating that the two techniques are comparable.

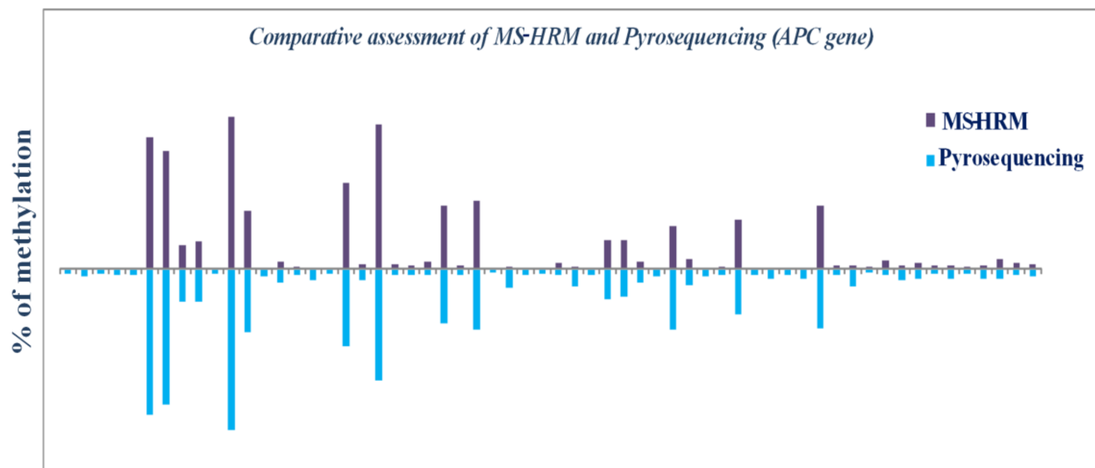


Figure 27: Specular profile of *APC* methylation (%) obtained for each subject with the two techniques.

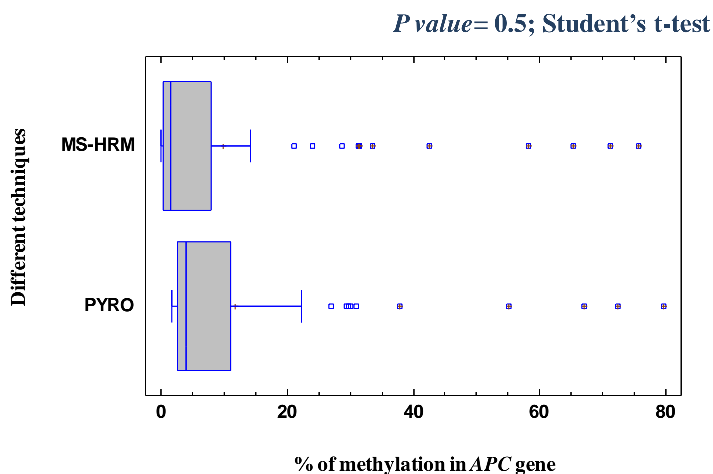


Figure 28: Comparison of mean values of *APC* methylation (%) using the two techniques.

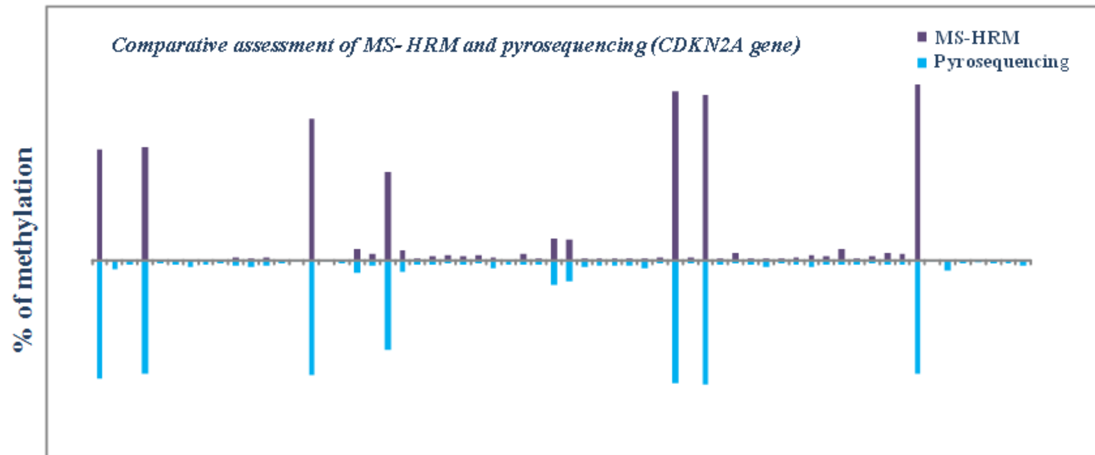


Figure 29: Specular profile of *CDKN2A* methylation (%) obtained for each subject with the two techniques.

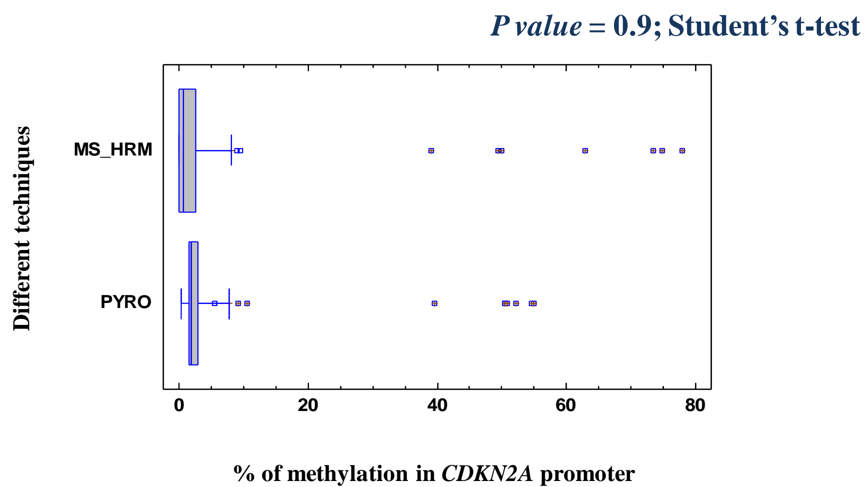


Figure 30: Comparison of mean values of *CDKN2A* methylation using the two techniques

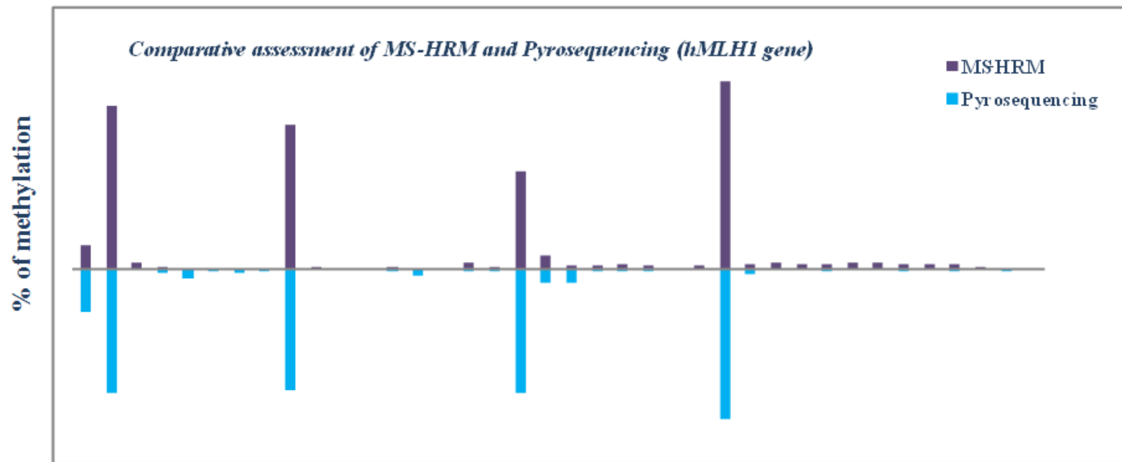


Figure 31: Specular profile of *hMLH1* methylation gene obtained for each subject with the two techniques.

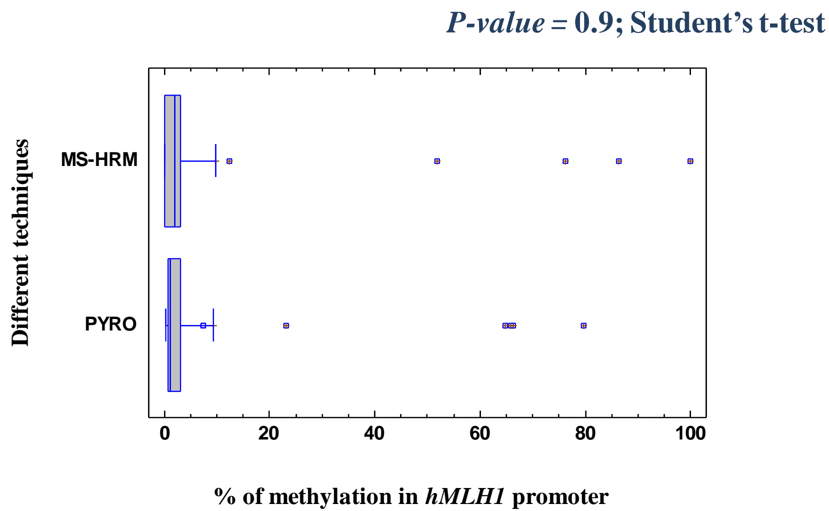


Figure 32: Comparison of mean values of *hMLH1* methylation using the two techniques

4. Tumor cells separation

Human epithelial cells were isolated from colorectal cancer and healthy tissues through immunomagnetic method with microbeads coated with the antibody CD326+, specific for epithelial cells. We expected CD326+ cells had a greater % of methylation respect to tissue without separation (CD326+ and CD326-). Data obtained with Pyrosequencing were thus analysed for 9 samples. There was no statistically significant difference between the epithelial cells and the tissue without separation in the promoter methylation levels of *APC, CDKN2A, MGMT* and *hMLH1* genes (Table 11), although few patients showed singularly a statistically significant difference between the two groups regarding the degree of methylation of these genes (data not reported). The table clearly shows that, on average, there was no significant difference in the levels of methylation of the selected genes between the two experimental conditions.

Table 11: Comparison of methylation levels (detected by Pyrosequencing) between whole tissue (without separation) and separated epithelial cells (CD326+) for four genes in 9 CRC patients.

Genes	**CD236+ cells (mean of methylation)**	**Cells without separation (mean of methylation)**	***p-value*** *
APC	19.61	17.02	0.1862
CDKN2A	14.75	12.91	0.469
hMLH1	17.67	16.7	0.82
MGMT	72.68	68.17	0.09

* *p-value* obtained by means ANOVA

5. Polymorphisms in folate metabolism genes and methylation

The CRC patients were genotyped for eight polymorphisms in one-carbon metabolism genes (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G, *TYMS* 28 bp repeats, *TYMS* 6 bp del, *DNMT3B* C-149T, *DNMT3B* G-579T). The distribution of the genotypes is provided in Table 12. In a subgroup (30 subjects) of our population, using data obtained by means of Pyrosequencing, we tested a possible correlation between both *MTHFR* C677T, *DNMT3B* C-149T and CpG sites methylation of *APC, CDKN2A*, *hMLH1* and *MGMT* promoter genes (Figures 33, 34, 35, 36, 37, 38, 39, 40). There is a statistically significant correlation (adjusted for age, gender, location, simple ID, size and stage) between the *MTHFR* genotypes and *APC* and *hMLH1* promoter methylation (*P value* = 0.0000 and *P value* = 0.003 respectively) (Figures 33, 35). For the other two genes there is not association with *MTHFR* C677T polymorphism (Figures 34, 36). Moreover there is a statistically significant association (adjusted for age, gender, location, sample ID, size and stage) between the *DNMT3B* genotypes and the *APC*, *CDKN2A*, *hMLH1* and *MGMT* promoter methylation (*P value* = 0.045, *P value* = 0.0000, *P value* = 0.006, *P value* = 0.0002, respectively) (Figures 37, 38, 39, 40).

Table 12: Genotypes relative to polymorphisms in one-carbon metabolism genes

Polymorphisms	Genotype frequencies		
***MTHFR* C677T**	CC: (37.1%)	CT: (46.8%)	TT: (16.1%)
***MTHFR* A1298C**	AA: (54.6%)	AC: (31.8%)	CC: (13.6%)
***MTR* A2756G**	AA: (78.1%)	AG: (21.9%)	GG: (0%)
***MTRR* A66G**	AA: (37.5%)	AG: (37.5%)	GG: (25%)
***TYMS* 28 bp repeats**	3R3R: (35.5%)	2R3R: (54.8%)	2R2R: (9.7%)
***TYMS* 6 bp del**	6+6+: (38%)	6+6-: (51.7%)	6-6-: (10.3%)
***DNMT3B* C-149T**	CC: (36.2%)	CT: (44.8%)	TT: (19%)
***DNMT3B* G-579T**	GG: (46.5%)	GT: (35.7%)	TT: (17.8%)

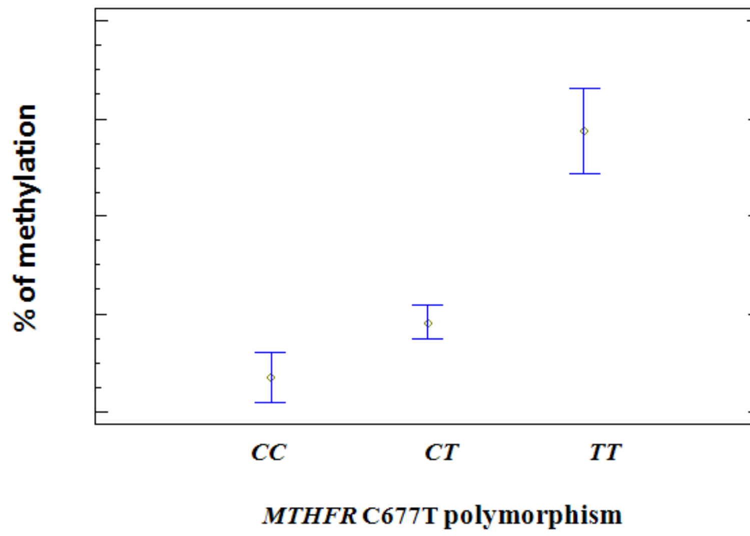


Figure 33: Correlation between *APC* methylation and *MTHFR* C677T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.0000).

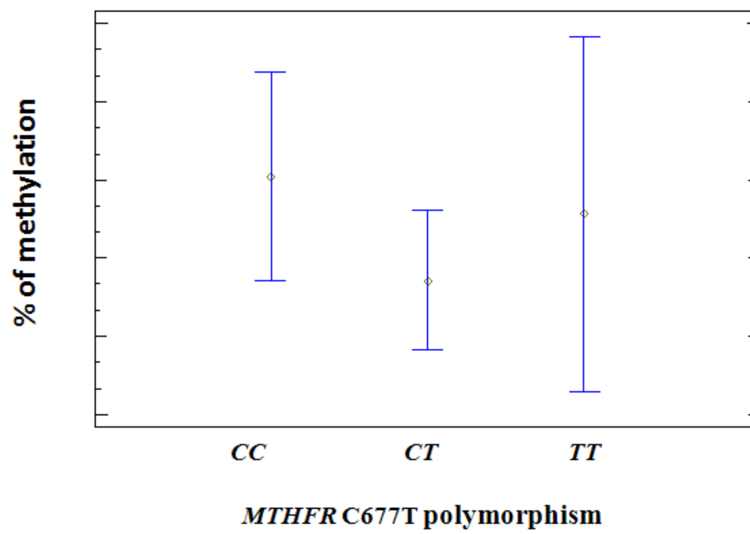


Figure 34: Correlation between *CDKN2A* methylation and *MTHFR* C677T polymorphism, adjusted for age, gender, location, sample ID, size and stage (P-value = 0.5).

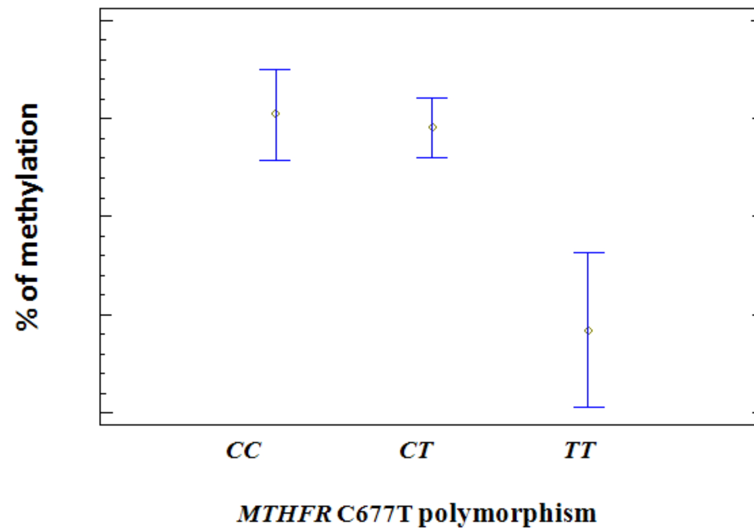


Figure 35: Correlation between *hMLH1* methylation and *MTHFR* C677T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.003).

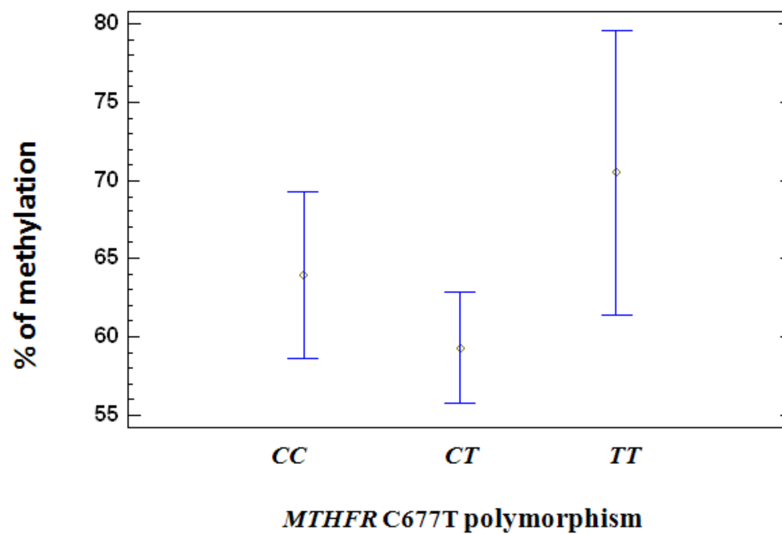


Figure 36: Correlation between *MGMT* (CpG island B) methylation and *MTHFR* C677T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.24).

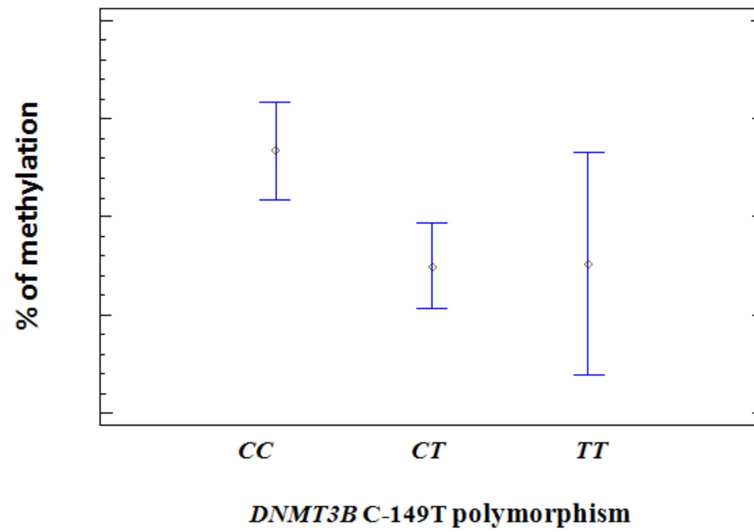


Figure 37: Correlation between *APC* methylation and *DNMT3B* C-149T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.045).

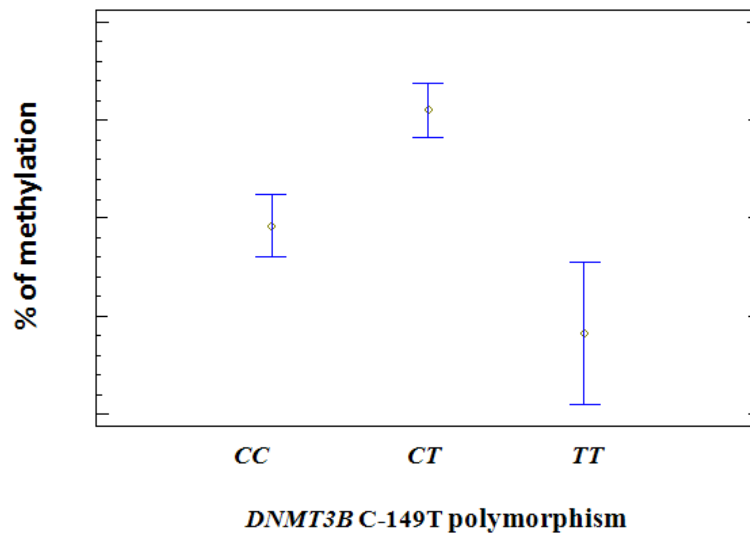


Figure 38: Correlation between *CDKN2A* methylation and *DNMT3B* C-149T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.0000).

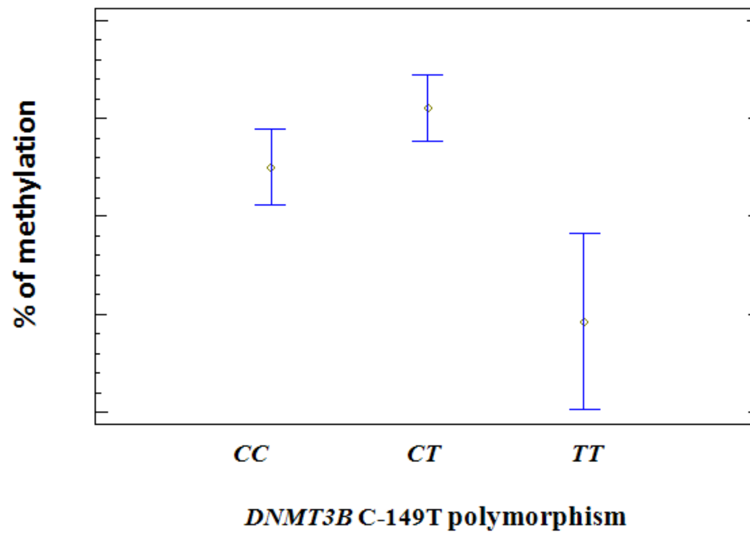


Figure 39: Correlation between *hMLH1* methylation and *DNMT3B* C-149T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.006).

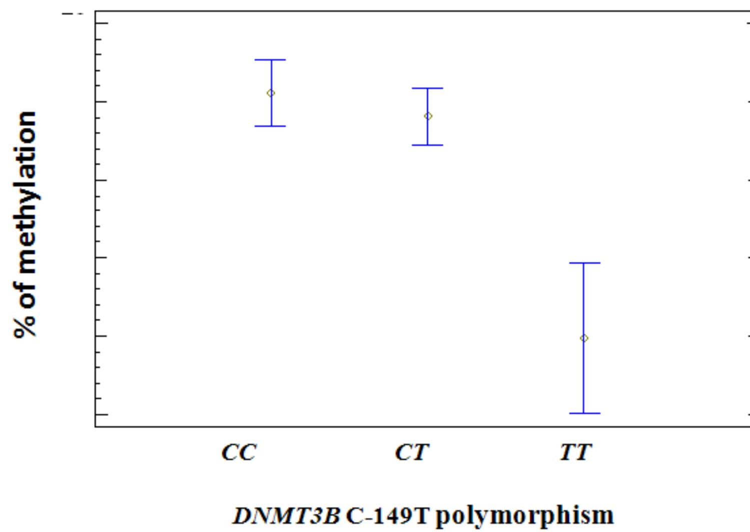


Figure 40: Correlation between *MGMT* methylation and *DNMT3B* C-149T polymorphism, adjusted for age, gender, location, sample ID, size and stage (*P-value* = 0.0002).

6. Folate, homocysteine and vitamin B12 values in CRC patients

From blood samples plasma folate, homocysteine and vitamin B12 have been measured. For some patients these biomarkers were not available, due to technical reasons. Interestingly, several CRC patients have high plasma Hcy levels (above the normal range that is 4.3-11.1 µmol/l) (Table 13).

Table 13: Folate, homocysteine and vitamin B12 values in CRC patients with the demographic characteristics of the subgroup.

	Age	Gender	Stage (TNM)	Tumor size	Hcy (µmol/l)	Folati (ng/ml)	Vit B12 (pg/ml)
P2	58	F	C (Stage III)	n.a	8,08	11,3	631
P3	42	M	B (Stage II)	T3	4,04	5,8	455
P4	79	M	n.a	n.a	17,67	7,3	523
P5	73	M	n.a.	n.a.	15,29	5,6	337
P6	86	F	B (Stage II)	T2	21,39	7,8	601
P7	78	F	n.a	n.a	11,86	8,2	612
P10	68	M	C (Stage III)	T3	7,48	n.a.	n.a.
P12	80	F	C (Stage III)	T3	6,54	n.a.	n.a.
P13	71	M	C (Stage III)	T3	20,44	6,1	203
P14	58	M	C (Stage III)	T3	10,56	8,1	441
P15	70	F	A(Stage 0)	Tis	n.a.	n.a.	403
$P16^{INK4A}$	71	F	A (Stage I)	T2	7,2	9,1	364
P17	47	F	C (Stage III)	T3	6,15	14	331
P18	77	M	A (Stage I)	T2	18,04	3,8	835
P23	48	M	B (Stage II)	T3	9,56	4,7	543
P27	77	M	B (Stage II)	T3	14,2	6,01	394
P28	64	F	A (Stage I)	T2	5,53	10,7	914
P41	62	F	C (Stage III)	T3	8,57	13,1	142
P45	87	F	B (Stage II)	T3	n.a.	4,5	410

P47	63	M	D (Stage IV)	T3	10,65	5	620
P49	50	F	B (Stage II)	T3	8,24	2,8	365

Discussion

The transformation of normal colon epithelial cells to adenomas, and then to cancer, is believed to be an evolutionary process in which neoplastic cells acquire heritable genetic and epigenetic alterations that drive the cancerogenesis (Migliore *et al.*, 2011). Gene promoter hypermethylation is now recognized to have an important role in cancer development through silencing gene transcription. It is also known that the genetic and epigenetic alterations cooperate to promote tumor formation; human cancers generally show global DNA hypomethylation accomplished by region-specific hypermethylation. Alterations of DNA methylation may result in chromosomal instability as a result of changes in chromatin structure. DNA hypermethylation of CpG sites silences various tumor-related genes. Numerous studies using candidate gene approaches and genome-wide screening techniques demonstrated that methylation-induced silencing of biologically relevant genes in colorectal cancer is common and influences tumor behavior (Hinoue *et al.*, 2012).

In the present thesis the methylation profiles of five CRC-related genes, namely *APC, MGMT, RASSF1A, hMLH1* and *CDKN2A* ($p16^{Ink4A}$) by means of MS-HRM were analyzed. Much effort was put on the validation of methodological approaches employed. First of all we set up MS-HRM protocol by comparing literature methodological indications focusing in particular on primer design. Some methylation studies use one primer set (MIP, methylation independent primer) to obtain a PCR product from bisulfite modified template regardless of its methylation status; however, proportional amplification of methylated and unmethylated alleles is difficult to obtain, due to PCR bias favoring amplification of the unmethylated sequence (poor in CpG). Wodjacz *et al* (2009, 2008) proposed a method to avoid this preferential amplification;

they suggested the inclusion of CpG sites into the primer sequence in fact the primers with limited number of CpG sites are able to reverse PCR bias and detect methylated templates with significantly higher sensitivity respect to CpG free primers. MS-HRM combines PCR amplification using methylation independent primers with subsequent HRM analyses of the PCR products; the PCR products generated from a methylated template have a higher GC content and therefore higher melting temperature respect to PCR products obtained from an unmethylated template. The fragment amplified during MS-HRM should be of a particular size: a short product will give high sensitivity but limited resolution between different levels of methylation because of the smaller differences in melting profiles between methylated and unmethylated products (Wodjacz *et al*, 2007). MS-HRM is an in tube method; thus the analysis takes place without the PCR product leaving the tube where it was amplified. This is important for the rapidity that it affords and because it could allow to avoid contamination. Then it was investigated if any significant difference was detectable when evaluating the methylation profile of the selected genes with MS-HRM, with respect to the methylation profile achievable by means of Pyrosequencing. Results obtained by means of MS-HRM matches those obtained with Pyrosequencing in terms of global DNA methylation of the studied amplicons, assuming that the same CpG sites are analysed within the CpG island. However we can compare the data of these two techniques only using the same set of primers. In fact, as already discussed, the primer design is a critical point for gene-specific methylation studies. Results from MS-HRM and Pyrosequencing have shown to be quantitatively comparable (see Figure 27, 28, 29, 30, 31, 32); the first technique is however a close-tube method, important condition to avoid possible contamination; moreover it is less expensive respect to Pyrosequencing; on the other hand this last method can give accurate information on the methylation

level regarding all the CpG sites collocated in the CpG island analyzed. MS-HRM technique results sensitive in particular when the samples contain a mixture of fully methylated and unmethyated templates, giving melting profiles comparable to standard melting curves; while in case of heterogeneous methylation the technique shows complex melting profiles, difficult to interpret. Quantification of heterogeneous methylation remains indeed difficult to detect; however these two techniques provide complementary information for the assessment of heterogeneous methylation because of the quantitative information.

Then, we questioned whether or not a selection of cancer epithelial cells (CD326+) cells from the surgically resection CRC tissue could give a more accurate result in terms of DNA methylation with respect to the whole CRC tissue. From data elaboration we did not observe statistically significant difference between the epithelial cells and the tissue without separation about the promoter methylation of *APC, CDKN2A, MGMT* and *hMLH1* genes (see Table 11), although some patients showed individually a statistically significant difference between the two experimental conditions regarding the degree of methylation of these genes (data not shown). It could be useful to analyse a larger tumor samples to verify the real importance of this method.

Concerning MS-HRM analysis we screened a total of 62 CRC samples obtained from the surgical resection of patients at different stages as well as 48 adjacent healthy colon tissue. *APC* resulted the most frequently methylated gene in our cohort, showing promoter methylation in almost 50% of CRC tissues. This is the only gene that showed also a low percentage of methylation (0-12.5%) in 39% of adjacent normal tissue (see Table 6). The *CDKN2A* gene showed promoter methylation in 26% of CRC tissues, while the corresponding adjacent tissue did not show altered levels of methylation (see Table 7). Concerning the *MGMT* gene we observed a 35% of tumor tissues methylated,

and also a weak methylation (0-12.5%) in 13% of adjacent normal tissues analyzed (see Table 10). *hMLH1* and *RASSF1A* are the least methylated genes in both tissues, found to be methylated in less than 15% of the analyzed samples (Tables 8, 9). The low levels of methylation observed in our study in normal appearing colorectal mucosa may be due to the circumscription of this phenomenon to limited area, rather than a spread diffuse alteration throughout the colon. We then performed a multivariate analysis of variance to test the effect of age, gender, cancer location, tumor size, and tumor stage to the levels of promoter methylation of each of the studied genes. In our cohort we did not find any statistically significant association between stage (TNM), gender, sex, tumor size, and location with regard to the methylation profile of each of the analyzed genes. However, we found an interesting positive association between age and both *hMLH1* (*P value*= 0.007) and *MGMT (P value= 0.03)* methylation (Figure 25, 26). Several investigators reported association between methylation of CRC-related genes in relation to one-carbon metabolites and polymorphisms of the genes involved in one-carbon metabolism (reviewed in Coppedè, 2011). Our investigation of the correlation between *MTHFR* 677C>T, *DNMT3B* -149C>T and gene promoter methylation revealed several significant interactions.

The maintenance of cell–cell interactions is crucial to avoid unregulated cell growth. The Apc protein plays an important role in cell adhesion via β-catenin. Since the *APC* gene is a tumor suppressor gene, both alleles should be inactivated for cancer development. These two independent events could be allelic imbalance, promoter hypermethylation or mutations. The analysis of *APC* methylation in DNA from tumor tissues and corresponding normal tissues of 5 FAP patients revealed hypermethylation of the gene in tumor tissues of one proband and her son. No methylation was present in

normal tissues. Loss of heterozygosity was observed in another patient from the same FAP family. Thus aberrant methylation of the *APC* promoter region provides an important mechanism for impairing APC function and may occur early during colon neoplasia progression (Zhang *et al.*, 2008). According to present results, another study revealed that the methylation status of *APC* had no significant association with clinical parameters (Naghibalhossaini *et al.*, 2011). According to Lao and Grady (2011) *MGMT* and *hMLH1* are frequently methylated in the polyp/adenoma → metastasis transition. Although we failed to find a significant effect of tumor stage to their promoter methylation profiles, the methylation levels of both of them showed a significant correlation with increasing age. According to our results, a previous study reported that the prevalence of *hMLH1* and *MGMT* methylation increases significantly with age (Menigatti *et al.*, 2009). Moreover, an association of *MGMT* promoter methylation with age and tumour grade was also observed in breast cancer tissues (Tserga *et al.*, 2012). Interestingly both genes are involved in DNA repair processes and their methylation might reflect an age-related decline of DNA repair capabilities. During the last years an increasing number of genes has been reported to be affected by methylation changes during aging. Up to 70% of CGIs found to be hypermethylated in primary colon tumors, was due to an age-related increase in hypermethylation of the normal colon; in the same way of the hypermethylation of specific CGIs, the genomewide and locus-specific loss of methylation occurs as a direct function of age (Wojdacz and Hansen, 2006).

$p16^{Ink4A}$ and *RASSF1A* are also frequently methylated in CRC tissues. According to Lao and Grady (2011) $p16^{Ink4A}$ is frequently methylated in the aberrant/crypt focus→ polyp/adenoma passage, and *RASSF1A* seems more methylated in the last stages or in metastases (see Migheli and Migliore, 2012). It is however noteworthy to say that none of these genes is stage specific and they are often methylated in different CRC stages, as

we observed in the present study. Here, no correlation among promoter methylation and CRC stage, location and size was observed, nor it was associated with patient age at sampling. Recent studies also suggest that CRC is not an unique disease in terms of DNA methylation, and at least 3-4 different CRC subtypes can be recognized according to high-low frequency of DNA methylation, chromosome instability and point mutations in selected genes (Hinoue *et al.*, 2012). This might also partially explain some of the conflicting results observed in the literature.

DNA methylation is largely depending from the availability of one-carbon nutrients and is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: *DNMT1, DNMT3A,* and *DNMT3B*. The *DNMT3B* gene, located on chromosome 20q11.2, contains a C-to-T transition polymorphism in the promoter region of the gene, -149 base pairs from the transcription start site. A case-control study reported that this polymorphism is associated with increased risk of CRC (Zhu *et al.*, 2012). Difference in the promoter activity of the variants could affect DNMT3B activity on DNA methylation, thereby modulating the susceptibility to cancer. In an *in vitro* assay this polymorphism was found significantly increase promoter activity of the *DNMT3B* gene. It was postulated that the T variant may up-regulate DNMT3B expression, resulting in a predisposition towards aberrant *de novo* methylation of CpG sites in tumor suppressor and DNA repair genes (Jair *et al.,* 2006).

We observed some positive correlations between the *DNMT3B* -149C>T polymorphism and the levels of methylation of *APC, CDKN2A, hMLH1* and *MGMT* genes (see Figures 37, 38, 39, 40). Albeit preliminary and obtained in a small group of patients our data are corroborated by previous findings by others suggesting that *DNMT3B* polymorphisms are associated with *p14ARF* methylation in CRC tissues. The authors investigated however a different polymorphism in the same gene (Kang *et al.*, 2008).

MTHFR is an enzyme that converts 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, the prevailing form of circulating folate and the methyl donor for the conversion of homo-cysteine to methionine. The 677T variant is associated with reduced enzyme activity (van der Put *et al.*, 1998), and the *MTHFR* 677TT genotype was associated with a lower risk (0.68) of colorectal cancer compared with the homozygous CC genotype (Lee *et al.*, 2012). Several previous studies suggest association between the *MTHFR* 677C>T polymorphism and gene methylation in CRC tissues, including *hMLH1, CDKN2A* genes (Mokarram *et al.*, 2008; Zhu *et al.*, 2011). There is also plenty of data on association between one-carbon nutrients (folate, vitamin B12, vitamin B6), their interaction with alcohol consumption, and DNA methylation in CRC (reviewed in Coppedè, 2011). Here, we observed significant associations between the *MTHFR* 677C>T polymorphisms and methylation of *hMLH1* and *APC* genes (Figure 33, 35). Interestingly, a study showed that in HCT116 cells, the *MTHFR* 677T mutation was associated with a significant increase in DNMT activity; this may be due to a compensatory response to the decreased SAM concentrations to maintain a critical level of DNA methylation (Batra *et al.*, 2007). Parallel to the observed increased DNMT activity, the *MTHFR* 677T mutation in HCT116 cells was associated with a significant increase in DNA methylation at sufficient or very high folate concentrations. At a low folate concentration, the *MTHFR* 677T mutation was associated with a significant reduction in DNA methylation, according to the observation that the *MTHFR* C677T polymorphism is associated with genomic DNA hypomethylation in human lymphocytes only with low folate intake (Sohn *et al.*, 2009; Castro *et al.*, 2004). These results indicate possible *MTHFR-DNMT* interactions and gene-nutrient interactions to be investigated as soon as we could collect a larger cohort of subjects.

Moreover, several interactions among age at sampling, gene polymorphisms and DNA methylation have been observed, deserving confirmation in a larger cohort of subjects.

Although the molecular mechanisms responsible for aberrant DNA methylation during carcinogenesis are still not well characterized, the implications of DNA hypomethylation and hypermethylation in the etiology of CRC tumorigenesis have become quite clear. Since epigenetic modifications are reversible, methylation studies are extremely promising to better characterize CRC and to identify new tools for diagnosis and prognosis. An interesting non-invasive method has been proposed for diagnostic evaluations of preclinical samples to detect cancer-derived methylation of vimentin gene in plasma and fecal DNA from colon cancer patients (Chen *et al.*, 2005, Li *et al.*, 2009). To date, a few pharmacological compounds directed toward epigenetic enzymes have shown promise in treating leukemias and lymphoma. These include DNA demethylating agents or (DNMT) inhibitors such as 5-azacytidine and 5-aza-20-deoxycytidine and HDACi such as suberoylanilide hydroxamic acid (SAHA). DNMT and HDACi are still being tested in metastatic CRC patients to improve the quality of life and survival of patients in the near future (Crea *et al.*, 2011). Interestingly a study showed an increase in the expression of DNMT1 in a colon tumor group of animals as compared to healty controls; moreover, the rats treated with 5-azacytidine alone or with combination of 5-azacytidine and cisplatin showed significant reduction in the expression of DNMT1, resulting in a protection against tumor growth (Tikoo *et al.*, 2009).

References

Abouzeid HE, Kassem AM, Abdel Wahab AH, El-mezayen HA, Sharad H, Abdel Rahman S. Promoter hypermethylation of RASSF1A, MGMT, and HIC-1 genes in benign and malignant colorectal tumors. Tumour Biol. 2011; 32: 845-852.

An B, Kondo Y, Okamoto Y, Shinjo K, Kanemitsu Y, Komori K, Hirai T, Sawaki A, Tajika M, Nakamura T, Yamao K, Yatabe Y, Fujii M, Murakami H, Osada H, Tani T, Matsuo K, Shen L, Issa JP, Sekido Y. Characteristic methylation profile in CpG island methylator phenotype-negative distal colorectal cancers. Int J Cancer. 2010; 127: 2095-2105.

Arasaradnam RP, Commane DM, Bradburn D, Mathers JC. A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. Epigenetics. 2008; 3: 193–198.

Ashktorab H, Belgrave K, Hosseinkhah F, Brim H, Nouraie M, Takkikto M, Hewitt S, Lee EL, Dashwood RH, Smoot D. Global histone H4 acetylation and HDAC2 expression in colon adenoma and carcinoma. Dig Dis Sci. 2009; 54: 2109-2117

Basten GP, Duthie SJ, Pirie L, Vaughan N, Hill MH, Powers HJ. Sensitivity of markers of DNA stability and DNA repair activity to folate supplementation in healthy volunteers. Br J Cancer. 2006; 94: 1942-1947.

Batra V, Mishra KP. Modulation of DNA methyltransferase profile by methyl donor starvation followed by gamma irradiation. Mol Cell Biochem. 2007; 294: 181–187.

Candiloro IL, Mikeska T, Dobrovic A. Assessing combined methylation-sensitive high resolution melting and pyrosequencing for the analysis of heterogeneous DNA methylation. Epigenetics. 2011; 6: 500-507.

Cunningham D, Atkin W, Lenz HJ et al. Colorectal cancer. Lancet 2010; 375: 1030–1047.

Carmona FJ, Esteller M. Moving closer to a prognostic DNA methylation signature in colon cancer. Clin Cancer Res. 2011; 17: 1215-1217.

Castro R, Rivera I, Ravasco P, Camilo ME, Jakobs C, Blom HJ, de Almeida IT. 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C-->T and 1298A-->C mutations are associated with DNA hypomethylation. J Med Genet. 2004; 41: 454–458.

Chang E, Park DI, Kim YJ, Kim BK, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Kim HD, Kim DH, Kim YH. Detection of colorectal neoplasm using promoter methylation of ITGA4, SFRP2, and $p16^{Ink4A}$ in stool samples: a preliminary report in Korean patients. Hepatogastroenterology. 2010; 57: 720-727.

Chen WD, Han ZJ, Skoletsky J, Olson J, Sah J, Myeroff L, Platzer P, Lu S, Dawson D, Willis J, Pretlow TP, Lutterbaugh J, Kasturi L, Willson JK, Rao JS, Shuber A, Markowitz SD. Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst. 2005; 97: 1124-1132.

Coppedè F, Marini G, Bargagna S, Stuppia L, Minichilli F, Fontana I, Colognato R, Astrea G, Palka G, Migliore L. Folate gene polymorphisms and the risk of Down syndrome pregnancies in young Italian women. Am J Med Genet A. 2006; 140: 1083-91.

Coppedè F, Zitarosa MT, Migheli F, Lo Gerfo A, Bagnoli S, Dardano A, Nacmias B, Mancuso M, Monzani F, Siciliano G, Sorbi S, Migliore L. DNMT3B promoter polymorphisms and risk of late onset Alzheimer's disease. Curr Alzheimer Res. 2012. [Epub ahead of print]

Coppedè F. Epigenetic biomarkers of colorectal cancer: Focus on DNA methylation. Cancer Lett. 2011. [Epub ahead of print]

Corfe BM, Williams EA, Bury JP, Riley SA, Croucher LJ, Lai DY, Evans CA. A study protocol to investigate the relationship between dietary fiber intake and fermentation, colon cell turnover, global protein acetylation and early carcinogenesis: the FACT study. BMC Cancer. 2009; 9: 332.

Crea F, Nobili S, Paolicchi E et al. Epigenetics and chemoresistance in colorectal cancer: an opportunity for treatment tailoring and novel therapeutic strategies. Drug Resist Updat 2011: 14: 280–296.

Curtin K, Slattery ML, Ulrich CM, Bigler J, Levin TR, Wolff RK, Albertsen H, Potter JD, Samowitz WS. Genetic polymorphisms in one-carbon metabolism: associations with CpG island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. Carcinogenesis. 2007; 28: 1672–1679.

De Vogel S, Dindore V, Van Engeland M, Goldbohm RA, Van Den Brandt PA, Weijenberg MP. Dietary folate, methionine, riboflavin, and vitamin B-6 and risk of sporadic colorectal cancer. J Nutr. 2008; 138: 2372–2378.

De Vogel S, Wouters KA, Gottschalk RW, van Schooten FJ, de Goeij AF, de Bruïne AP, Goldbohm RA, van den Brandt PA, Weijenberg MP, van Engeland M. Genetic variants of methyl metabolizing enzymes and epigenetic regulators: associations with promoter CpG island hypermethylation in colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2009; 18: 3086-3096.

Devos T, Tetzner R, Model F, Weiss G, Schuster M, Distler J, Steiger KV, Grützmann R, Pilarsky C, Habermann JK, Fleshner PR, Oubre BM, Day R, Sledziewski AZ, Lofton-Day C. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clin Chem. 2009; 55: 1337–1346.

Du W, Li WY, Lu R, Fang JY. Folate and fiber in the prevention of colorectal cancer: between shadows and the light. World J Gastroenterol. 2010; 16: 921-926.

Dumitrescu RG. Epigenetic markers of early tumor development. Methods Mol Biol. 2012; 863: 3-14.

Duthie SJ, Pirie LP, Grant G, Watson AJ, Margison GP. Long term folate deficiency differentially alters hepatic and colon MGMT and OGG-1 DNA repair protein expression in rats but has no impact on genome-wide DNA methylation. Cancer Prev Res (Phila). 2010; 3: 92-100.

Duthie SJ. Epigenetic modifications and human pathologies: cancer and CVD. Proc Nutr Soc. 2011; 70: 47-56.

Eaton AM, Sandler R, Carethers JM, Millikan RC, Galanko J, Keku TO. 5,10-Methylenetetrahydrofolate reductase 677 and 1298 polymorphisms, folate intake, and microsatellite instability in colon cancer. Cancer Epidemiol Biomarkers Prev. 2005; 14: 2023-2029.

Esteller M. Epigenetic changes in cancer. F1000 Biol Rep 2011; 3: 9.

Esteller M. Epigenetics in evolution and disease. Lancet. 2008; 372: 590-596.

Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990; 61: 759-67.

Fu X, Li J, Li K, Tian X, Zhang Y. Hypermethylation of APC promoter 1A is associated with moderate activation of Wnt signalling pathway in a subset of colorectal serrated adenomas. Histopathology. 2009; 55: 554-63.

Gay LJ, Arends MJ, Mitrou PN, Bowman R, Ibrahim AE, Happerfield L, Luben R, McTaggart A, Ball RY, Rodwell SA. MLH1 Promoter Methylation, Diet, and Lifestyle Factors in Mismatch Repair Deficient Colorectal Cancer Patients From EPIC-Norfolk. Nutr Cancer. 2011; 63: 1000-10.

Grady WM, Parkin RK, Mitchell PS, Lee JH, Kim YH, Tsuchiya KD, Washington MK, Paraskeva C, Willson JK, Kaz AM, Kroh EM, Allen A, Fritz BR, Markowitz SD, Tewari M. Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer Oncogene. 2008; 27: 3880-8.

Grützmann R, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, Miehlke S, Stolz T, Model F, Roblick UJ, Bruch HP, Koch R, Liebenberg V, Devos T, Song X, Day RH, Sledziewski AZ, Lofton-Day C. Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA methylation assay. PLoS One. 2008; 3: e3759.

Halford S, Rowan A, Sawyer E, Talbot I, Tomlinson I. O(6)-methylguanine methyltransferase in colorectal cancers: detection of mutations, loss of expression, and weak association with G:C>A:T transitions. Gut. 2005; 54:797-802.

Hamid A, Kiran M, Rana S, Kaur J. Low folate transport across intestinal basolateral surface is associated with down-regulation of reduced folate carrier in in vivo model of folate malabsorption. IUBMB Life. 2009; 61: 236-243.

Han HB, Gu J, Zuo HJ, Chen ZG, Zhao W, Li M, Ji DB, Lu YY, Zhang ZQ. Let-7c functions as a metastasis suppressor by targeting MMP11 and PBX3 in colorectal cancer. J Pathol. 2012; 226: 544-55.

He Q, Chen HY, Bai EQ, Luo YX, Fu RJ, He YS, Jiang J, Wang HQ. Development of a multiplex MethyLight assay for the detection of multigene methylation in human colorectal cancer. Cancer Genet. Cytogenet. 2010; 202: 1-10.

Hibi K, Goto T, Mizukami H, Kitamura Y, Sakata M, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y. MGMT gene is aberrantly methylated from the early stages of colorectal cancers. Hepatogastroenterology. 2009; 56: 1642-4.

Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res. 2012; 22: 271-82.

Hirsch S, Sanchez H, Albala C, de la Maza MP, Barrera G, Leiva L, Bunout D. Colon cancer in Chile before and after the start of the flour fortification program with folic acid. Eur J Gastroenterol Hepatol 2009; 21(4):436–439.

Huang KT, Dobrovic A, Yan M, Karim RZ, Lee CS, Lakhani SR, Fox SB. DNA methylation profiling of phyllodes and fibroadenoma tumours of the breast. Breast Cancer Res. 2010; 124: 555-65.

Hughes LA, van den Brandt PA, de Bruïne AP, Wouters KA, Hulsmans S, Spiertz A, Goldbohm RA, de Goeij AF, Herman JG, Weijenberg MP, van Engeland M. Early life exposure to famine and colorectal cancer risk: a role for epigenetic mechanisms. PLoS One. 2009; 4: e7951.

Huidobro C, Urdinguio RG, Rodríguez RM, Mangas C, Calvanese V, Martínez-Camblor P, Ferrero C, Parra-Blanco A, Rodrigo L, Obaya AJ, Suárez-Fernández L, Astudillo A, Hernando H, Ballestar E, Fernández AF, Fraga MF. A DNA methylation signature associated with aberrant promoter DNA hypermethylation of DNMT3B in human colorectal cancer. Eur J Cancer. 2012. [Epub ahead of print]

Iacopetta B, Heyworth J, Girschik J, Grieu F, Clayforth C, Fritschi L. The MTHFR C677T and DNMT3B C-149T polymorphisms confer different risks for right- and left-sided colorectal cancer. Int J Cancer. 2009; 125: 84-90.

Isaksson-Mettävainio M, Palmqvist R, Dahlin AM, Van Guelpen B, Rutegård J, Oberg A, Henriksson ML. High SMAD4 levels appear in MSI and hypermethylated colon cancers, and indicate a better prognosis. Int J Cancer. 2011 [Epub ahead of print].

Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, Henning SM, Swendseid ME. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr. 1998; 128: 1204-1212.

Jair KW, Bachman KE, Suzuki H, Ting AH, Rhee I, Yen RW, Baylin SB, Schuebel KE. De novo CpG island methylation in human cancer cells. Cancer Res. 2006; 66: 682-692.

Jasperson KW, Tuohy TM, Neklason DW, Burt RW. “Hereditary and familial colon cancer”. Gastroenterology. 2010; 138: 2044-2058.

Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007; 128:683-692.

Kang MY, Lee BB, Ji YI, Jung EH, Chun HK, Song SY, Park SE, Park J, Kim DH. Association of interindividual differences in p14ARF promoter methylation with single nucleotide polymorphism in primary colorectal cancer. Cancer. 2008; 112: 1699-1707.

Kang S, Kim JW, Kang GH, Park NH, Song YS, Kang SB, Lee HP. Polymorphism in folate- and methionine-metabolizing enzyme and aberrant CpG island hypermethylation in uterine cervical cancer. Gynecol Oncol. 2005; 96: 173-180.

Kang YP, Cao FA, Chang WJ, Lou Z, Wang H, Wu LL, Fu CG, Cao GW. Gene methylation in stool for the screening of colorectal cancer and pre-malignant lesions. Zhonghua Wei Chang Wai Ke Za Zhi. 2011; 14: 52-56.

Karpinski P, Myszka A, Ramsey D, Misiak B, Gil J, Laczmanska I, Grzebieniak Z, Sebzda T, Smigiel R, Stembalska A, Sasiadek MM. Polymorphisms in methyl-group metabolism genes and risk of sporadic colorectal cancer with relation to the CpG island methylator phenotype. Cancer Epidemiol. 2010; 34: 338-344.

Keku T, Millikan R, Worley K, Winkel S, Eaton A, Biscocho L, Martin C, Sandler R. 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. Cancer Epidemiol Biomarkers Prev. 2002; 11: 1611-1621.

Kim MS, Chung NG, Kang MR, Yoo NJ, Lee SH. Genetic and expressional alterations of CHD genes in gastric and colorectal cancers. Histopathology 2011; 58: 660-668.

Kim MS, Lee J, Sidransky D. DNA methylation markers in colorectal cancer. Cancer Metastasis Rev. 2010; 29: 181-206.

Kim YI. Folate and colorectal cancer: an evidence-based critical review. Mol Nutr Food Res. 2007; 51: 267-292.

Kim YI. Folic acid supplementation and cancer risk: point. Cancer Epidemiol Biomarkers Prev. 2008; 17: 2220-2225.

Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S, Mori M. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res. 2011; 71: 6320-6326.

Krakowczyk L, Strzelczyk JK, Adamek B, Zalewska-Ziob M, Arendt J, Półtorak S, Maciejewski B, Wiczkowski A. Methylation of the MGMT and p16^{Ink4A} genes in sporadic colorectal carcinoma and corresponding normal colonic mucosa. Med Sci Monit. 2008; 14: BR219-25.

Lao VV, Grady WMEpigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol. 2011; 18; 8: 686-700.

Lara E, Calvanese V, Huidobro C, Fernández AF, Moncada-Pazos A, Obaya AJ, Aguilera O, González-Sancho JM, Sánchez L, Astudillo A, Muñoz A, López-Otín C, Esteller M, Fraga MF. Epigenetic repression of ROR2 has a Wnt-mediated, pro-tumourigenic role in colon cancer. Mol Cancer. 2010; 9: 170.

Lee BB, Lee EJ, Jung EH, Chun HK, Chang DK, Song SY, Park J, Kim DH. Aberrant methylation of APC, MGMT, RASSF2A, and Wif1 genes in plasma as a biomarker for early detection of colorectal cancer. Clin Cancer Res 2009; 15: 6185-6191.

Lee JE, Wei EK, Fuchs CS, Hunter DJ, Lee IM, Selhub J, Stampfer MJ, Willett WC, Ma J, Giovannucci E. Plasma folate, methylenetetrahydrofolate reductase (MTHFR), and colorectal cancer risk in three large nested case-control studies. Cancer Causes Control. 2012. [Epub ahead of print].

Lee JS, Nam JH, Choi C, Lee MC, Park CS, Juhng SW, Lee JH. Promoter methylation status of hMLH1, hMSH2, and MGMT genes in colorectal cancer associated with adenoma-carcinoma sequence. Langenbecks Arch Surg. 2011; 396: 1017-1026.

Leong KJ, Wei W, Tannahill LA Caldwell GM, Jones CE, Morton DG, Matthews GM, Bach SP. Methylation profiling of rectal cancer identifies novel markers of early-stage disease. Br J Surg. 2011; 98: 724-734.

Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg. 2002; 89: 845-860.

Levine AJ, Siegmund KD, Ervin CM, Diep A, Lee ER, Frankl HD, Haile RW. The methylenetetrahydrofolate reductase 677C→T polymorphism and distal colorectal adenoma risk," Cancer Epidemiol Biomarkers Prev. 2000; 9: 657-663.

Li M, Chen W, Papadopoulos N et al. Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 2009: 27: 858–863.

Lind GE, Danielsen SA, Ahlquist T, Merok MA, Andresen K, Skotheim RI, Hektoen M, Rognum TO, Meling GI, Hoff G, Bretthauer M, Thiis-Evensen E, Nesbakken A, Lothe RA. Identification of an epigenetic biomarker panel with high sensitivity and specificity for colorectal cancer and adenomas. Mol Cancer. 2011; 10: 85.

Lindzon GM, Medline A, Sohn KJ, Depeint F, Croxford R, Kim YI. Effect of folic acid supplementation on the progression of colorectal aberrant crypt foci. Carcinogenesis. 2009; 30: 1536-1543.

Liu M, Chen H. The role of microRNAs in colorectal cancer. J Genet Genomics. 2010; 37: 347-358.

Liu X, Zhang Z, Sun L, Chai N, Tang S, Jin J, Hu H, Nie Y, Wang X, Wu K, Jin H, Fan D. MicroRNA-499-5p promotes cellular invasion and tumor metastasis in colorectal cancer by targeting FOXO4 and PDCD4. Carcinogenesis. 2011; 32: 1798–1805.

Lopez-Serra P, Esteller M. DNA methylation-associated silencing of tumor suppressor microRNAs in cancer. Oncogene. 2011; [Epub ahead of print].

Madrigano J, Baccarelli A, Mittleman MA, Wright RO, Sparrow D, Vokonas PS, Tarantini L, Schwartz J. Prolonged exposure to particulate pollution, genes

associated with glutathione pathways and DNA methylation in a cohort of older men. Environ Health Perspect. 2011; 119: 977-982.

Mason JB, Dickstein A, Jacques PF, Haggarty P, Selhub J, Dallal G, Rosenberg IH. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomark Prev. 2007; 16: 1325-1329.

Menigatti M, Truninger K, Gebbers JO, Marbet U, Marra G, Schär P. Normal colorectal mucosa exhibits sex- and segment-specific susceptibility to DNA methylation at the hMLH1 and MGMT promoters. Oncogene. 2009; 28: 899-909.

Michor F, Iwasa Y, Lengauer C, Nowak MA. Dynamics of colorectal cancer. Semin Cancer Biol. 2005; 15: 484-493.

Migliore L, Migheli F, Spisni R, Coppedè F. Genetics, cytogenetics, and epigenetics of colorectal cancer. J Biomed Biotechnol. 2011; 2011: 792362.

Migheli F, Migliore L.Clin Genet. Epigenetics of colorectal cancer. 2012; 81: 312-8.

Miladi-Abdennadher I, Abdelmaksoud-Damak R, Ayadi L, Khabir A, Frikha F, Kallel L, Frikha M, Sellami-Boudawara T, Gargouri A, Mokdad-Gargouri R Aberrant methylation of hMLH1 and p16^{Ink4AInk4A} in Tunisian patients with sporadic colorectal adenocarcinoma. Biosci Rep. 2011; 31: 257-264.

Mokarram P, Kumar K, Brim H, Naghibalhossaini F, Saberi-firoozi M, Nouraie M, Green R, Lee E, Smoot DT, Ashktorab H. Distinct high-profile methylated genes in colorectal cancer. PLoS One. 2009; 4: e7012.

Mokarram P, Naghibalhossaini F, Saberi Firoozi M, Hosseini SV, Izadpanah A, Salahi H, Malek-Hosseini SA, Talei A, Mojallal M. Methylenetetrahydrofolate reductase C677T genotype affects promoter methylation of tumor-specific genes

in sporadic colorectal cancer through an interaction with folate/vitamin B12 status. World J Gastroenterol. 2008; 14: 3662-71.

Mossman D, Scott RJ. Long term transcriptional reactivation of epigenetically silence genes in colorectal cancer cell s requires DNA hypomethylation and histone acetlation. PLoS One 2011; 6: e23127.

Nagasaka T, Goel A, Notohara K, Takahata T, Sasamoto H, Uchida T, Nishida N, Tanaka N, Boland CR, Matsubara N. Methylation pattern of the O6-methylguanine-DNA methyltransferase gene in colon during progressive colorectal tumorigenesis. Int J Cancer. 2008; 122: 2429-2436.

Naghibalhossaini F, Hosseini HM, Mokarram P, Zamani M. High frequency of genes' promoter methylation, but lack of BRAF V600E mutation among Iranian colorectal cancer patients. Pathol Oncol Res. 2011; 17: 819-825.

Naghibalhossaini F, Zamani M, Mokarram P, Khalili I, Rasti M, Mostafavi-Pour Z. Epigenetic and genetic analysis of WNT signaling pathway in sporadic colorectal cancer patients from Iran. Mol Biol Rep. 2011; [Epub ahead of print].

Nakayama G, Kodera Y, Ohashi N, Koike M, Fujiwara M, Nakao A. $p16^{Ink4AInk4A}$ methylation in serum as a follow-up marker for recurrence of colorectal cancer. Anticancer Res. 2011; 31: 1643-1646.

Nakazawa T, Kondo T, Ma D, Niu D, Mochizuki K, Kawasaki T, Yamane T, Iino H, Fujii H, Katoh R. Global histone modification of histone H3 in colorectal cancer and its precursor lesion. Hum Pathol. 2011; [Epub ahead of print].

Nosho K, Kawasaki T, Ohnishi M, Suemoto Y, Kirkner GJ, Zepf D, Yan L, Longtine JA, Fuchs CS, Ogino S.PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. Neoplasia. 2008; 10: 534-541.

Nystrom M, Mutanen M. Diet and epigenetics in colon cancer. World J Gastroenterol. 2009; 15: 257-263.

Ogino S, Kawasaki T, Kirkner GJ, Suemoto Y, Meyerhardt YA, Fuchs CS. Molecular correlates with MGMT promoter methylation and silencing support CpG island methylator phenotype-low (CIMP-low) in colorectal cancer. Gut. 2007; 56: 1564-1571.

Olivo-Marston SE, Hursting SD, Lavigne J, Perkins SN, Maarouf RS, Yakar S, Harris CC. Genetic reduction of circulating insulin-like growth factor- 1 inhibits azoxymethane-induced colon tumorigenesis in mice. Mol Carcinog. 2009; 48: 1071-1076.

Paun BC, Kukuruga D, Jin Z, Mori Y, Cheng Y, Duncan M, Stass SA, Montgomery E, Hutcheon D, Meltzer SJ. Relation between normal rectal methylation, smoking status, and the presence or absence of colorectal adenomas. Cancer. 2010; 116: 4495-4501.

Phelip JM, Ducros V, Faucheron JL, Flourie B, Roblin X. Association of hyperhomocysteinemia and folate deficiency with colon tumors in patients with inflammatory bowel disease. Inflamm Bowel Dis. 2008; 14(2):242-248.

Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. Gastroenterology. 2010; 138: 2059-2072.

Piyathilake C, Badiga S, Johanning G, Alvarez R, Partridge E. Predictors and health consequences of epigenetic changes associated with excess body weight in women of child-bearing age. Cancer Epidemiol Biomarkers Prev 2011; 20: 719.

Psofaki V, Kalogera C, Tzambouras N, Stephanou D, Tsianos E, Seferiadis K, Kolios G. Promoter methylation status of hMLH1, MGMT, and CDKN2A/p16^{Ink4A} in colorectal adenomas. World J Gastroenter 2010; 16: 3553-3560.

Rampersaud GC, Kauwell GP, Hutson AD, Cerda JJ, Bailey LB. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. Am J Clin Nutr. 2000; 72: 998-1003.

Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Mol Oncol. 2007; 1: 19-25.

Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 2008; 116: 1547-1552.

Sameer AS, Abdullah S, Nissar S, Rasool R, Shah ZA, Afroze D, Chowdri NA, Siddiqi MA. The blues of P(16)INK(4a): Aberrant promoter methylation and association with colorectal cancer in the Kashmir valley. Mol Med Report. 2012; 5: 1053-1057.

Schernhammer ES, Giovannucci E, Kawasaki T, Rosner B, Fuchs CS, Ogino S. Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. Gut. 2010; 59(6):794-799.

Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP. MGMT promoter methylation and field defect in sporadic colorectal cancer. J Natl Cancer Inst. 2005; 97: 1330-1338.

Shima K, Morikawa T, Baba Y, Nosho K, Suzuki M, Yamauchi M, Hayashi M, Giovannucci E, Fuchs CS, Ogino S. MGMT promoter methylation, loss of expression and prognosis in 855 colorectal cancers. Cancer Causes Control. 2011; 22: 301-309.

Sie KK, Medline A, van Weel J, Sohn KJ, Choi SW, Croxford R, Kim YI. Effect of maternal and postweaning folic acid supplementation on colorectal cancer risk in in the offspring. Gut 2011; 60: 1687-1694.

Slattery ML, Curtin K, Sweeney C, Levin TR, Potter J, Wolff RK, Albertsen H, Samowitz WS. Diet and lifestyle factor associations with CpG island methylator phenotype and BRAF mutations in colon cancer. Int J Cancer. 2007; 120: 656-663.

Slattery ML, Potter JD, Samowitz W, Schaffer D, Leppert M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol Biomarkers Prev. 1999; 8: 513-518.

Sohn KJ, Jang H, Campan M, Weisenberger DJ, Dickhout J, Wang YC, Cho RC, Yates Z, Lucock M, Chiang EP, Austin RC, Choi SW, Laird PW, Kim YI. The methylenetetrahydrofolate reductase C677T mutation induces cell-specific changes in genomic DNA methylation and uracil misincorporation: a possible molecular basis for the site-specific cancer risk modification. Int J Cancer. 2009;124: 1999-2005.

Song Y, Xu Y, Wang Z Chen Y, Yue Z, Gao P, Xing C, Xu H. MicroRNA-148b suppresses cell growth by targeting cholecystokinin-2 receptor in colorectal cancer. Int J Cancer. 2011; [Epub ahead of print].

Suzuki K, Suzuki I, Leodolter A, Alonso S, Horiuchi S, Yamashita K, Perucho M. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell. 2006; 9: 199-207.

Svrcek M, Buhard O, Colas C, Coulet F, Dumont S, Massaoudi I, Lamri A, Hamelin R, Cosnes J, Oliveira C, Seruca R, Gaub MP, Legrain M, Collura A, Lascols O, Tiret E, Fléjou JF, Duval A Methylation tolerance due to an O6-methylguanine DNA

methyltransferase (MGMT) field defect in the colonic mucosa: an initiating step in the development of mismatch repair-deficient colorectal cancers. Gut. 2010; 59: 1516-1526.

Takeda M, Nagasaka T, Dong-Sheng S, Nishie H, Oka T, Yamada E, Mori Y, Shigeyasu K, Morikawa T, Mizobuchi S, Fujiwara T. Expansion of CpG methylation in the SFRP2 promoter region during colorectal tumorigenesis. Acta Med Okayama. 2011; 65: 169-77.

Tang JT, Wang JL, Du W, Hong J, Zhao SL, Wang YC, Xiong H, Chen HM, Fang JY. MicroRNA 345, a methylation sensitive microRNA is involved in cell proliferation and invasion in human colorectal cancer. Carcinogenesis 2011; 32: 1207-1215.

Tarantini L, Bonzini M, Apostoli P, Pegoraro V, Bollati V, Marinelli B, Cantone L, Rizzo G, Hou L, Schwartz J, Bertazzi PA, Baccarelli A. Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ Health Perspect. 2009; 117: 217-222.

Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. DNA methylation in white blood cells: association with risk factors in epidemiologic studies. Epigenetics. 2011; 6: 828-37.

Tikoo K, Ali IY, Gupta J, Gupta C. 5-Azacytidine prevents cisplatin induced nephrotoxicity and potentiates anticancer activity of cisplatin by involving inhibition of metallothionein, pAKT and DNMT1 expression in chemical induced cancer rats. Toxicol Lett 2009: 191: 158–166.

Tommasi S, Pinto R, Petriella D, Pilato B, Lacalamita R, Santini D, Zito F, Colucci G, Paradiso A, Silvestris N. Oncosuppressor methylation: a possible key role in colon metastatic progression. J Cell Physiol. 2011; 226: 1934-9.

Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc. 2007; 2: 2265-2275.

Tserga A, Michalopoulos NV, Levidou G, Korkolopoulou P, Zografos G, Patsouris E, Saetta AA. Association of aberrant DNA methylation with clinicopathological features in breast cancer. Oncol Rep. 2012; 27: 1630-1638.

Van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet. 1998; 62: 1044-1051.

Van Guelpen B, Hultdin J, Johansson I, Hallmans G, Stenling R, Riboli E, Winkvist A, Palmqvist R. Low folate levels may protect against colorectal cancer. Gut. 2006; 55: 1461-1466.

Wallace K, Grau MV, Levine AJ, Shen L, Hamdan R, Chen X, Gui J, Haile RW, Barry EL, Ahnen D, McKeown-Eyssen G, Baron JA, Issa JP. Association between folate levels and CpG Island hypermethylation in normal colorectal mucosa. Cancer Prev Res (Phila). 2010; 3: 1552-1564.

Wang F, Zhang P, Ma Y et al. NIRF is frequently upregulated in colorectal cancer and its oncogenicity can be suppressed by let-7a microRNA. Cancer Lett. 2012; 314: 223–231.

Wang YC, Yu ZH, Liu C, Xu LZ, Yu W, Lu J, Zhu RM, Li GL, Xia XY, Wei XW, Ji HZ, Lu H, Gao Y, Gao WM, Chen LB. Detection of RASSF1A promoter

hypermethylation in serum from gastric and colorectal adenocarcinoma patients. World J Gastroenterol. 2008; 14: 3074-80.

Wang Z, Yuan X, Jiao N, Zhu H, Zhang Y, Tong J. CDH13 and FLBN3 gene methylation are associated with poor prognosis in colorectal cancer. Pathol Oncol Res. 2011; [Epub ahead of print].

Wasson GR, McGlynn AP, McNulty H, O'Reilly SL, McKelvey-Martin VJ, McKerr G, Strain JJ, Scott J, Downes CS. Global DNA and p53 region-specific hypomethylation in human colonic cells is induced by folate depletion and reversed by folate supplementation. J Nutr. 2006; 136: 2748-2753.

Wei Q, Shen H, Wang LE. Association between low dietary folate intake and suboptimal cellular DNA repair capacity. Cancer Epidemiol Biomarker Prev. 2003; 12: 963-969.

Wojdacz TK, Borgbo T, Hansen LL. Primer design versus PCR bias in methylation independent PCR amplifications. Epigenetics. 2009; 4: 231-234.

Wojdacz TK, Dobrovic A, Hansen LL. Methylation-sensitive high-resolution melting. Nat Protoc. 2008; 3: 1903-1908.

Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. Nucleic Acids Res. 2007; 35: e41.

Wojdacz TK, Hansen LL. Techniques used in studies of age-related DNA methylation changes. Ann N Y Acad Sci. 2006; 1067: 479-87.

Xiong H, Du W, Zhang YJ, Hong J, Su WY, Tang JT, Wang YC, Lu R, Fang JY. Trichostatin A, a histone deacetylase inhibitor, suppresses JAK2/STAT3 signaling via inducing the promoterassociated histone acetylation of SOCS1 and SOCS3 in human colorectal cancer cells. Mol Carcinog 2012; 51: 174-184.

Yamaji T, Iwasaki M, Sasazuki S, Sakamoto H, Yoshida T, Tsugane S. Methionine synthase A2756G polymorphism interacts with alcohol and folate intake to influence the risk of colorectal adenoma. Cancer Epidemiol Biomarkers Prev. 2009;18: 267-274.

Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci USA. 2008; 105: 13421-13426.

Yang XJ, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene 2007; 26: 5310-5318.

Yi JM, Dhir M, Van Neste L, Downing SR, Jeschke J, Glöckner SC, de Freitas Calmon M, Hooker CM, Funes JM, Boshoff C, Smits KM, van Engeland M, Weijenberg MP, Iacobuzio-Donahue CA, Herman JG, Schuebel KE, Baylin SB, Ahuja N.Genomic and epigenomic integration identifies a prognostic signature in colon cancer. Clin Cancer Res. 2011; 17: 1535-1545.

Zeisel SH. Gene response elements, genetic polymorphisms and epigenetics influence the human dietary requirement for choline. IUBMB Life. 2007; 59(6):380-387.

Zhang FF, Cardarelli R, Carroll J, Zhang S, Fulda KG, Gonzalez K, Vishwanatha JK, Morabia A, Santella RM. Physical activity and global genomic DNA methylation in a cancer-free population. Epigenetics. 2011a; 6: 293-299.

Zhang H, Widegren E, Wang DW, Sun HF. SPARCL1: a potential molecule associated with tumor diagnosis, progression and prognosis of colorectal cancer. Tumor Biol. 2011; 32: 1225–1231.

Zhang YY, Chen SQ, Zhu M, Li JT, Ma GJ, Zhang XM, Zhou JN. Promoter hypermethylation and loss of heterozygosity of the APC gene in patients with familial adenomatous polyposis. Zhonghua Yi Xue Yi Chuan Xue Za Zhi. 2008; 25: 378-81.

Zhu Q, Jin Z, Yuan Y, Lu Q, Ge D, Zong M. Impact of MTHFR gene C677T polymorphism on Bcl-2 gene methylation and protein expression in colorectal cancer. Scand J Gastroenterol. 2011; 46: 436-445.

Zhu S, Zhang H, Tang Y, Liu P, Wang J. DNMT3B polymorphisms and cancer risk: a meta analysis of 24 case-control studies. Mol Biol Rep. 2012; 39: 4429-37.

www.cancer.gov

www.genomatix.de

http://globocan.iarc.fr

List of papers produced whitin the PhD Program:

1. Coppedè F, Zitarosa MT, Migheli F, Lo Gerfo A, Bagnoli S, Dardano A, Nacmias B, Mancuso M, Monzani F, Siciliano G, Sorbi S, Migliore L. DNMT3B promoter polymorphisms and risk of late onset Alzheimer's disease. Curr Alzheimer Res. 2012; [Epub ahead of print].
2. Migheli F, Migliore L. Epigenetics of colorectal cancer. Clin Genet. 2012; 81: 312-8.
3. Coppedè F, Tannorella P, Pezzini I, Migheli F, Ricci G, Lenco EC, Piaceri I, Polini A, Nacmias B, Monzani F, Sorbi S, Siciliano G, Migliore L. Folate, Homocysteine, Vitamin B12, and Polymorphisms of Genes Participating in One-Carbon Metabolism in Late-Onset Alzheimer's Disease Patients and Healthy Controls. Antioxid Redox Signal. 2011; [Epub ahead of print].
4. Migliore L, Migheli F, Spisni R, Coppedè F; Genetics, cytogenetics, and epigenetics of colorectal cancer. J Biomed Biotechnol. 2011; 2011: 792362.
5. Coppedè F, Grossi E, Migheli F, Migliore L. Polymorphisms in folate-metabolizing genes, chromosome damage, and risk of Down syndrome in Italian women: identification of key factors using artificial neural networks. BMC Med Genomics 2010; 3: 42.
6. Coppedè F, Ceravolo R, Migheli F, Fanucchi F, Frosini D, Siciliano G, Bonuccelli U, Migliore L. The hOGG1 Ser326Cys polymorphism is not associated with sporadic Parkinson's disease. Neurosci Lett. 2010; 473: 248-51.
7. Coppedè F, Migheli F, Ceravolo R, Bregant E, Rocchi A, Petrozzi L, Unti E, Lonigro R, Siciliano G, Migliore L. The hOGG1 Ser326Cys polymorphism and Huntington's disease. Toxicology. 2010; 278: 199-203.

8. Migliore L, Migheli F, Coppedè F. Susceptibility to aneuploidy in young mothers of Down syndrome children. ScientificWorldJournal. 2009; 9: 1052-60.
9. Coppedè F, Migheli F, Lo Gerfo A, Fabbrizi MR, Carlesi C, Mancuso M, Corti S, Mezzina N, del Bo R, Comi GP, Siciliano G, Migliore L. Association study between XRCC1 gene polymorphisms and sporadic amyotrophic lateral sclerosis. Amyotroph Lateral Scler. 2010; 11: 122-4.
10. Coppedè F, Migheli F, Bargagna S, Siciliano G, Antonucci I, Stuppia L, Palka G, Migliore L. Association of maternal polymorphisms in folate metabolizing genes with chromosome damage and risk of Down syndrome offspring. 2008 Neurosci Lett. 449: 15-9.

Co-authored abstracts for National and International meetings:

1. F Migheli, F Coppedè; A Stoccoro, A Failli, G Gagliardi, G Orsini, R Consolini, R Spisni, L Migliore. Epigenetic analysis of colorectal carcinoma (CRC) related genes by means of Methylation Sensitive High-Resolution Melting (MS-HRM) in CRC patients. 19° Meeting of the Italian Environmental Mutagen Society (SIMA) Parma, September 28-30, 2011.
2. L Migliore, F Migheli, F Coppedè, A Failli, G Gagliardi, A Legittimo, G Orsini, R Spisni. Methylation-sensitive high-resolution melting (MS-HRM) analysis in the promoter of MGMT, APC, hMLH1, RASSF1A, CDKN2A genes in colorectal carcinoma. 41st European Environmental Mutagen Society Meeting, Barcelona (Espana), July 4-7, 2011.
3. F Migheli, F Coppedè, E Tognocchi, G Orsini, R Consolini, R Spisni, P Miccoli, L Migliore: High-resolution melting (HRM) analysis of MGMT promoter methylation in

colorectal carcinoma. Abstract XIII Congresso Nazionale SIGU. Firenze, 14-17 Ottobre 2010.

4. F. Migheli, F.Coppedè, E. Tognocchi, G.Orsini, R. Consolini, R. Spisni, P. Miccoli and L. Migliore. High-Resolution Melting (HRM) analysis of *MGMT* promoter methylation in colorectal carcinoma. 13th Congress of the Italian Society of Human Genetics (S.I.G.U.), October 14-17, 2010, Florence, Italy. Poster.
5. F. Coppedè, E. Grossi, F. Migheli, L. Migliore. Polymorphisms in folate metabolizing genes, chromosome damage, and risk of down syndrome: identification of key factors using artificial neural networks. 13th Congress of the Italian Society of Human Genetics (S.I.G.U.), October 14-17, 2010, Florence, Italy. Poster.
6. F. Coppedè, F. Migheli, M.T. Zitarosa, I. Pezzini, A. Lo Gerfo, I. Piaceri, B. Nacmias, M. Mancuso, G. Siciliano, S. Sorbi, L. Migliore. Polymorphisms in the DNA methyltransferase 3b (*DNMT3B*) gene and risk of late onset Alzheimer's disease. 13th Congress of the Italian Society of Human Genetics (S.I.G.U.), October 14-17, 2010, Florence, Italy. Poster.
7. I. Pezzini, F. Migheli, E. Caldarazzo Jenco, G. Ricci, S. Bagnoli, B. Nacmias, S. Sorbi, G. Siciliano, F. Coppedè, L. Migliore. Plasma folate, homocysteine and vitamin b12 values, and polymorphisms of folate metabolic genes in alzheimer's disease patients and controls. 13th Congress of the Italian Society of Human Genetics (S.I.G.U.), October 14-17, 2010, Florence, Italy. Poster.
8. F. Coppedè, F. Fanucchi, R. Ceravolo, F. Migheli, E. Unti, L. Petrozzi, M. Mancuso, G. Siciliano, L. Migliore (2009). Lack of association between the *OGG1* Ser326Cys polymorphism and risk for sporadic Parkinson's disease. 12th Congress of the Italian Society of Human Genetics (S.I.G.U.), November 8-10, 2009, Torino, Italy, Poster.

9. F. Coppedè, M.R. Fabbrizi, F. Migheli, A. Lo Gerfo, C. Carlesi, M. Mancuso, S. Corti, N. Mezzina, R. del Bo, G.P. Comi, G. Siciliano, L. Migliore (2009). Association between the *XRCC1* Arg399Gln polymorphism and risk for sporadic amyotrophic lateral sclerosis. 12th Congress of the Italian Society of Human Genetics (S.I.G.U.), November 8-10, 2009, Torino, Italy, Poster.
10. F. Migheli, R. Ceravolo, E. Bregant, A. Rocchi, L. Petrozzi, E. Unti, R. Lonigro, G. Siciliano, F. Coppedè, L. Migliore (2009). The *OGG1* Ser326Cys polymorphism and Huntington's disease. 12th Congress of the Italian Society of Human Genetics (S.I.G.U.), November 8-10, 2009, Torino, Italy.
11. F. Coppedè, F. Migheli, A. Lo Gerfo, R. Ceravolo, M. Mancuso, R del Bo, R. Lonigro, G.P. Comi, G. Siciliano, L. Migliore (2009). Polymorphisms in DNA base excision repair (BER) genes and neurodegenerative diseases.10th International Conference on environmental Mutagens. Florence, Italy, August 20-25, 2009. Poster.
12. F. Coppedè, F. Migheli, S. Bargagna, G. Siciliano, I. Antonucci, L. Stuppia, G. Palka, L. Migliore (2008). Association of maternal polymorphisms in folate metabolizing genes with chromosome damage, and with risk of down syndrome offspring. 11th Congress of the Italian Society of Human Genetics (S.I.G.U.), November 23-25, 2008, Genova, Italy, Poster.
13. L. Migliore, F. Coppedè, R. Colognato, F. Migheli, V. Sarli, A. Bonelli, G. Astrea, S. Bargagna, G. Siciliano. Polymorphisms in Folate Metabolising Genes and Micronuclei in Mothers of Down Syndrome Children. Abstracts of the 7th European Cytogenetics Conference, Istanbul, Turkey, June 4-7, abstract published in Chromosome Research (2007) 13, Supplement 1, Springer EDS, pages 105-106.
14. F. Coppedè, I. Fontana, F. Migheli, L. Stuppia, S. Bargagna, R. Colognato, G. Palka, L. Migliore. (2005) Folate gene polymorphisms and the risk of Down syndrome

pregnancies in young Italian women. 7th Congress of the Italian Federation of Life Sciences (FISV), September 22-25, 2005, Riva del Garda, Italy. Poster.

15. F. Coppedè, F. Migheli, C. Natola, L. Stuppia, S. Bargagna, F. Minichilli, I. Fontana, R. Colognato, G. Palka, L. Migliore. (2005) Folate metabolism and the risk of Down syndrome pregnancies in young Italian women. 35th Annual Meeting. European Environmental Mutagen Society. July 3-7, 2005, Kos Island, Greece. Poster.