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CELLULAR AND MOLECULAR BIOMARKERS FOR RISK ASSESSMENT OF COLORECTAL CANCER

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Index

Abstract

Colorectal Cancer (CRC) has been described as the third most common form of cancer among men and the second one among women in European Union. The detection of CRC, at early stages, is one of the proven strategies resulting in a higher cure rate and, although, CRC is one in the small group of cancers which can be prevented by screening, only 39% of cases are diagnosed at early stages. In recent years, several studies have appeared identifying potential cancer markers in serum, plasma and stool in an attempt to improve actual screening procedures. The measurement of MN frequency in peripheral blood lymphocytes has been successfully used to identify dietary and genetic factors which have a significant impact on genome stability and the utility as a prediction cancer risk tool is well documented. On the other hand, it has been demonstrated that epigenetic events, altogether with genetic events, plays a crucial role in tumor progression. DNA methylation is the most common epigenetic mechanism and consists in a covalent modification of DNA. Thus, the aim of the study was (1) Evaluate MN frequency, (2) Evaluate plasma ultrafiltrate capacity to induce MN formation, (3) Evaluate SEPT9 and NOTCH3 promoter methylation profile in peripheral blood lymphocytes from subjects resulted positive to fecal occult blood test and examined by colonoscopy.

MN frequency was significantly higher in subjects with histological diagnosis of CRC and adenoma than control ($p \leq 0.001$ and $p \leq 0.01$, respectively). Also, a statistically significant difference in MN frequency was observed between subjects with CRC and adenoma ($p \leq 0.001$). Relative Risk associated with MN frequency, age, gender, BMI and smoke habit, indicate that an elevated MN frequency leads to a 71% increased risk to develop adenoma and when associated with an elevated age leads to a 113% increased risk for CRC, considering control group as reference. About, CF-MN analysis, a statistically significant difference was observed between CRC and control ($p \leq 0.05$). On the other hand, SEPT9 and NOTCH3 promoter methylation status was significantly lower in CRC subjects than controls; additionally, NOTCH3 promoter methylation status was significantly lower in CRC subjects than adenoma subjects ($p \leq 0.01$).

The results obtained allow conclude that MN frequency varies according CRC pathologic status and, together with other variables, is a valid biomarker for adenoma and CRC risk. Additionally, the plasma of patients affected with CRC not only serve as a biomarker for oxidative stress but also as biomarker of genetic damage correlated with the carcinogenic process that verifies in colon-rectum. SEPT9 and NOTCH3 promoter methylation status, at peripheral blood level, varies according hystopathological changes observed in colon-rectum, suggesting that promoter methylation profile of these genes could be a reliable biomarker for CRC risk.

Abbreviations

APC	Adenomatous Polyposis Coli gene
BCL-2	B-cell lymphoma 2 gene
CIMP	CpG Island Methylatior Phenotype
CIN	Chromosomal Instability
CpG	C-phosphate-G
CRC	Colorectal Cancer
FAP	Familial Adenomatous Polyposis
FOBT	Fecal Occult Blood Test
Globocan	Global Cancer report
HNPCC	Hereditary NonPolyposis Colorectal Cancer
MLH	MutL Homolog gene
MN	Micronuclei
MSH	MutS Homolog gene
MSI	Microsatellite Instability
MSP	Methylation Specific PCR
MYC	Myelocytomatosis Oncogene
NEUROG1	Neurogenin 1 gene
PMS	Postmeiotic segregation increased
RAS	Rat Sarcoma gene
SEPT9	Septin 9 gene
SSRs	Simple Sequences Repeats
TNF-α	Tumor Necrosis Factor α
TNM	Tumor, Nodules, Metastasis

1. BACKGROUND

1.1 Cancer

1.1.1 General Considerations

The term cancer refers to several pathologies characterized by an incontrollable growth and diffusion of abnormal cells. Cancer is a complex pathology highly variable, regarding evolution and outcome, from one individual to another (Merlo *et al.* 2006). Even when it's an old pathology, it has become a main issue due to the human population growth rate and the increased life length expectation, which implies a longer period of exposure to carcinogenic agents.

The American Society of Cancer describes cancer as the second leading cause of death in economically developed countries and the third leading cause of death in developing countries (Garcia *et al.* 2007). For 2008 worldwide cancer cases were estimated in over 12.6 million and cancer-related death closed to 7.5 million, 20.000 deaths a day (Figure 1 and 2). By 2030, it is expected to grow to 21 million new worldwide cancer cases and 17.5 million of deaths (Globocan 2008).

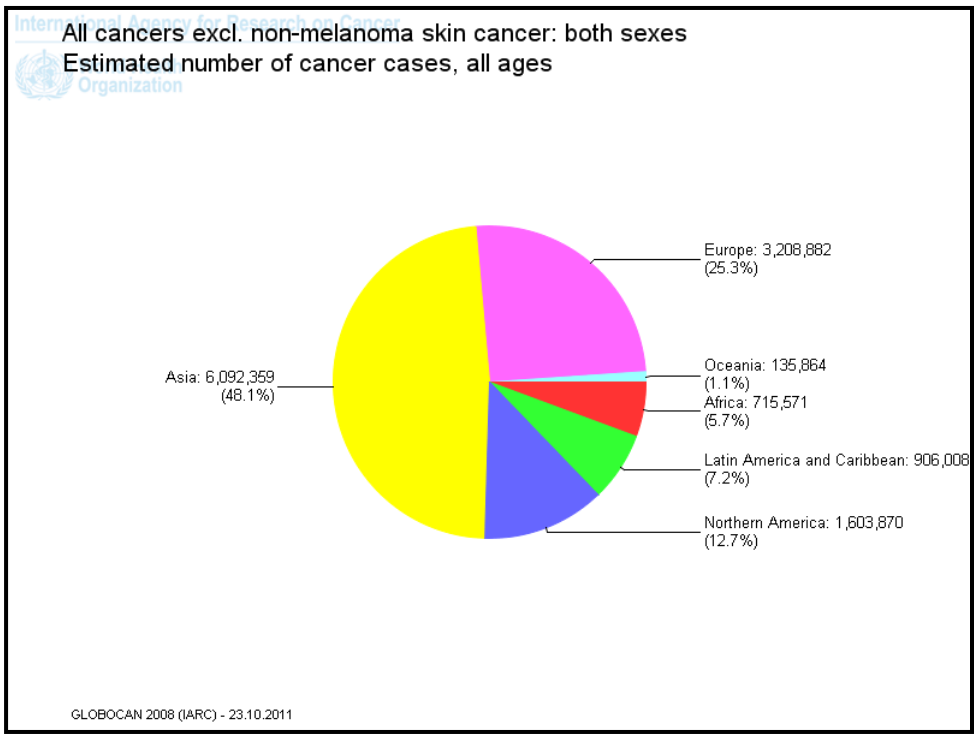

Figure 1. World cancer incidence by continent (Globocan 2008).

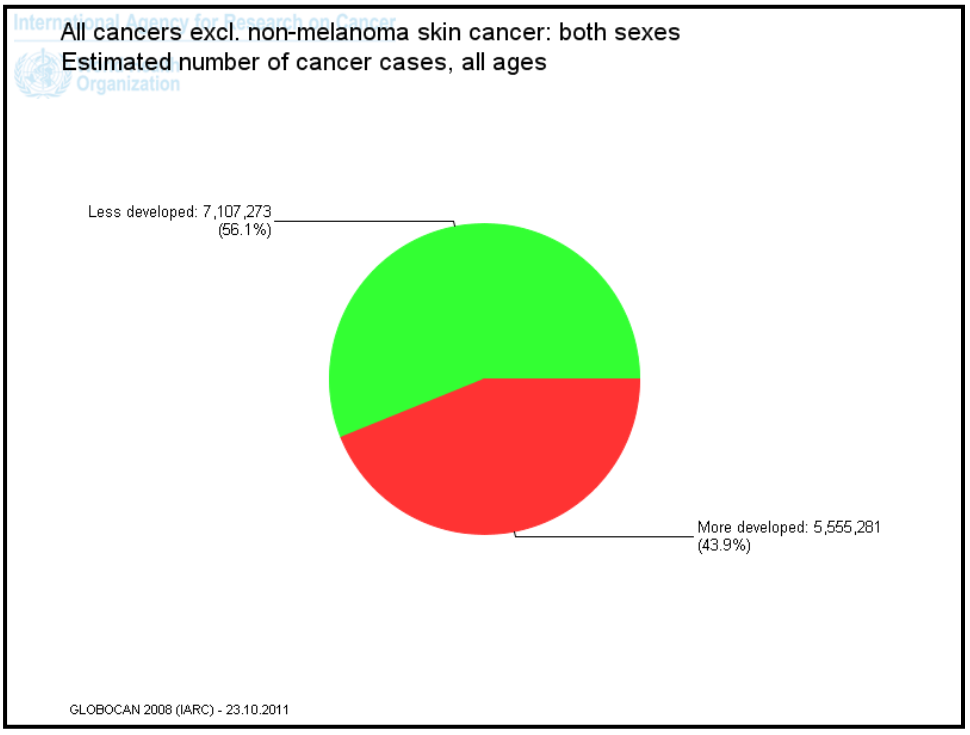

Figure 2. World cancer incidence by development status (Globocan 2008).

Risk factors associated with cancer appearance are strictly correlated with life style. This is how smoke has been related with different cancers like lung, oral cavity, pharynx, larynx and stomach. In developed countries, smoke has been indicated as responsible of 30% of all kind of cancers. Worldwide, smoke has been described as the most common source of carcinogen agents, particularly polycyclic aromatic hydrocarbons and nitrosamines (IARC 2004). In the same way, alcohol intake could also favor oral cavity, pharynx, larynx, liver, colon, rectum and breast cancer (Boffetta and Hashibe 2006). Diet has always been suggested as a possible factor that increases the risk of cancer, and it is expected that epidemiological studies may establish a clear relation between this factor and increased or decreased cancer risk. However, several studies have shown that, except for gastrointestinal cancer types, fat intake, fruit and vegetables, and meat intake are not correlated with other types of cancer, like breast or prostate (World Cancer Research Fund 2007). Additionally, it is well accepted that work exposure to different carcinogens like asbestos, polycyclic aromatic hydrocarbons, heavy metals, silica and ionizing radiations are associated with an increased cancer risk (IARC 2000, Louis *et al.* 2007).

On the other hand, it's important to highlight that 15 - 20% worldwide cancer cases have an infectious origin. Hepatocellular carcinoma caused by hepatitis B or C virus; cervix cancer caused by human papilloma virus; lymphoma caused by Epstein-Barr virus; stomach cancer caused by Helicobacter pilori, are examples of this particular group (World Cancer Report 2008).

Figure 3 shows the relative contribution of main risk factors according to the country develop status.

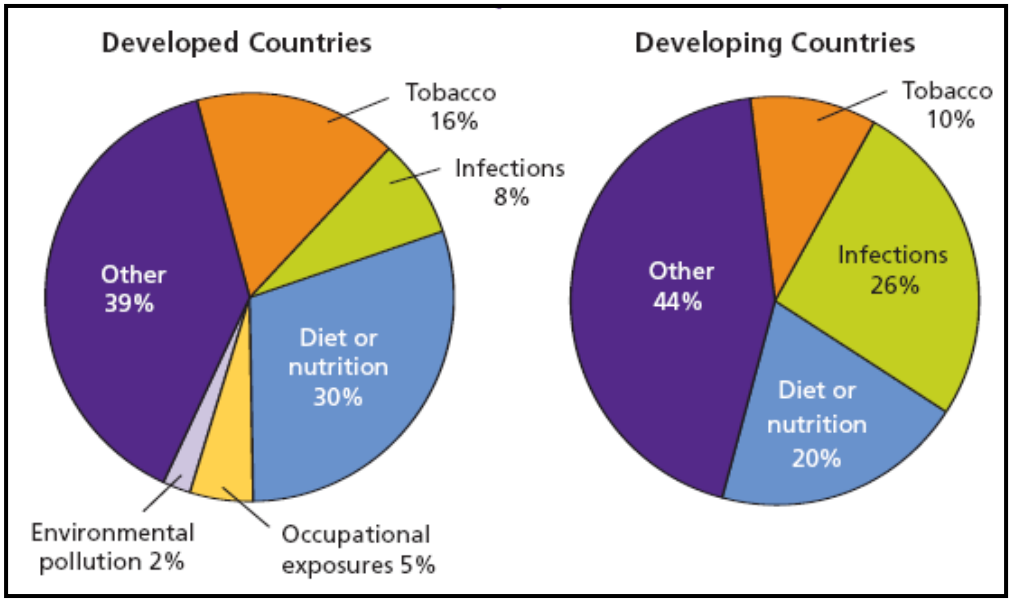

Figure 3. Main cancer risk factors by country develop status (Cancer Atlas 2006).

1.1.2 Tumor development

Cancer develops from one cell or a small group of cells which must undergo several mutations by specific genes (proto-oncogenes and oncosupressor genes) in order to turn cancerous. Proto-oncogenes, like RAS, MYC, BCL-2, are generally inactive but after several mutations may result activated favoring neoplastic cells proliferation (Rajagopalen *et al.* 2003, Sieber *et al.* 2003). On the other hand, same mutation of oncosupressor genes, like p53, which under normal conditions blocks cell division and induces apoptosis, may cause a loss of gene function leading to cell proliferation (Jallepalli and Lengauer 2001, Karaman *et al.* 2008). Indeed, one of the first capabilities acquired by neoplastic cells is to grow uncontrollably inducing abnormal cell cloning able to avoid immune system surveillance (Miyamoto and Rosenberg 2011).

Cancer is defined as a progressive pathology. It begins with a small benign lesion which remains confined in the original tissue, but in the presence of favorable conditions it may evolve into a malignant formation able to spread to surrounding tissues and organs, and through blood and lymphatic vessels colonize distant organs to form metastases.

Three phases are distinguishable during tumor formation: initiation, promotion and

progression. During the first one, pre-mutational lesions are produced due to the interaction between carcinogens and DNA in an irreversible and dose-independent way. If this damage is not repaired before a new division occurs, a mutation may be established, that compromises critical genome areas, like proto-oncogenes or oncosupressor genes previously mentioned. The second stage, promotion, is characterized by cellular hyperproliferation and may be caused by molecules other than those in initiation stage. A promoter catalyzes biochemical reactions in both normal and initiated cells, but only in the last ones it allows the expression of a new phenotype. This stage is reversible and dose-dependent. In the last stage, progression, the benign phenotype changes and becomes malignant with tumor manifestation (WHO 2000, World Cancer Report 2008).

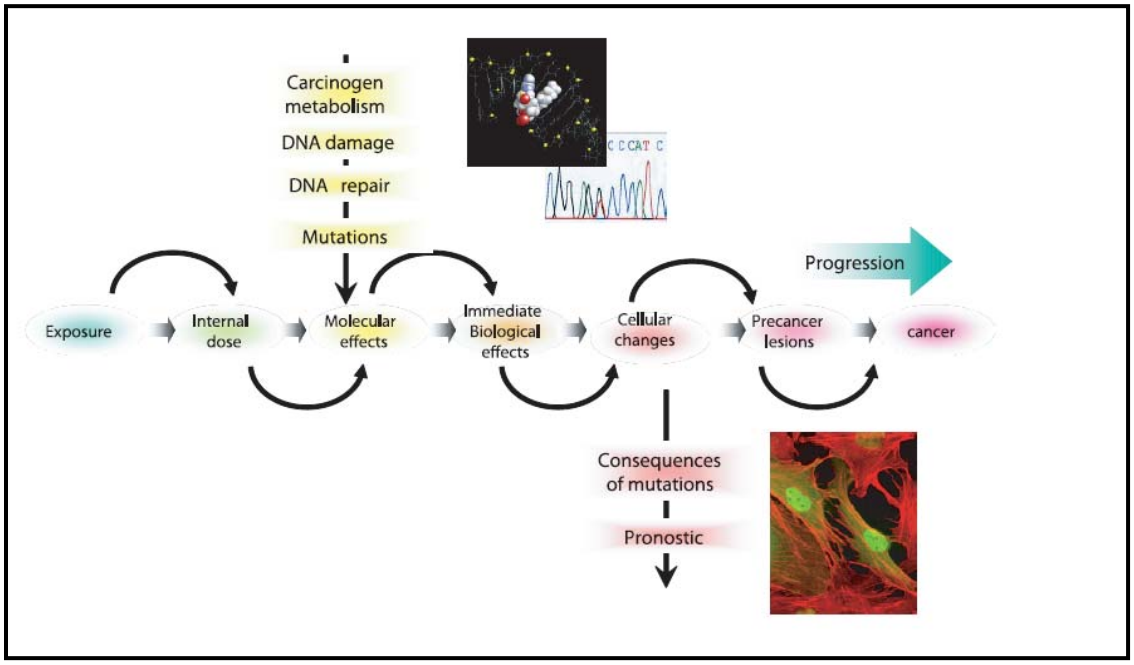

Figure 4. Cancer development: form risk factors to pathology. (World Cancer Report 2008).

1.2 Colorectal Cancer

1.2.1 Epidemiology

Of different types of cancer, Colorectal Cancer (CRC) has been described as the third most common form of cancer among men and the second one among women in the European Union (Globocan 2008), and one of the leading causes of cancer-related deaths in USA and Europe (Bolognesi *et al.* 2005) (Figure 5). However, CRC is a world spread disease strongly related to life style associated with countrie development situation (Rutter *et al.* 2006, Bonassi *et al.* 2007, Ferlay *et al.* 2007, Garcia *et al.* 2007, World Cancer Research Fund 2007) (Figure 6a and 6b).

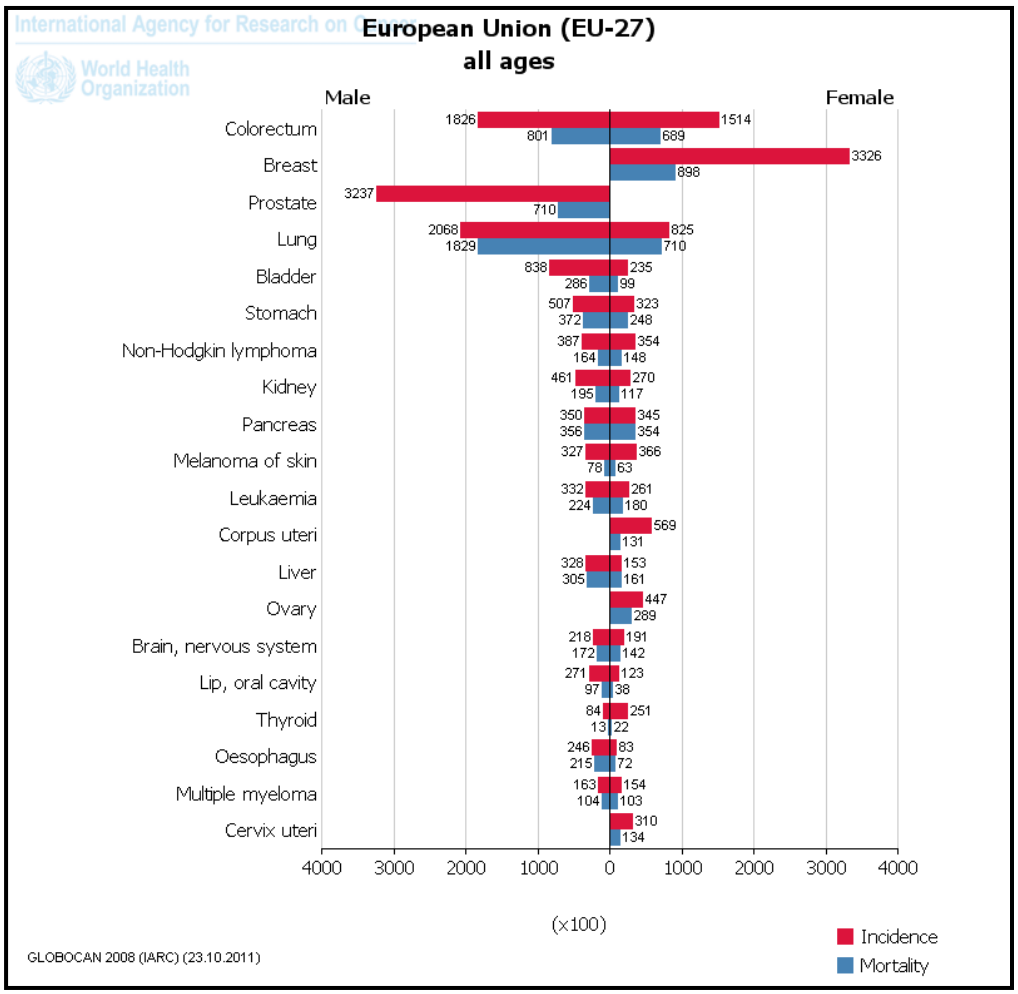

Figure 5. Incidence and mortality by cancer in European Union (Globocan 2008)

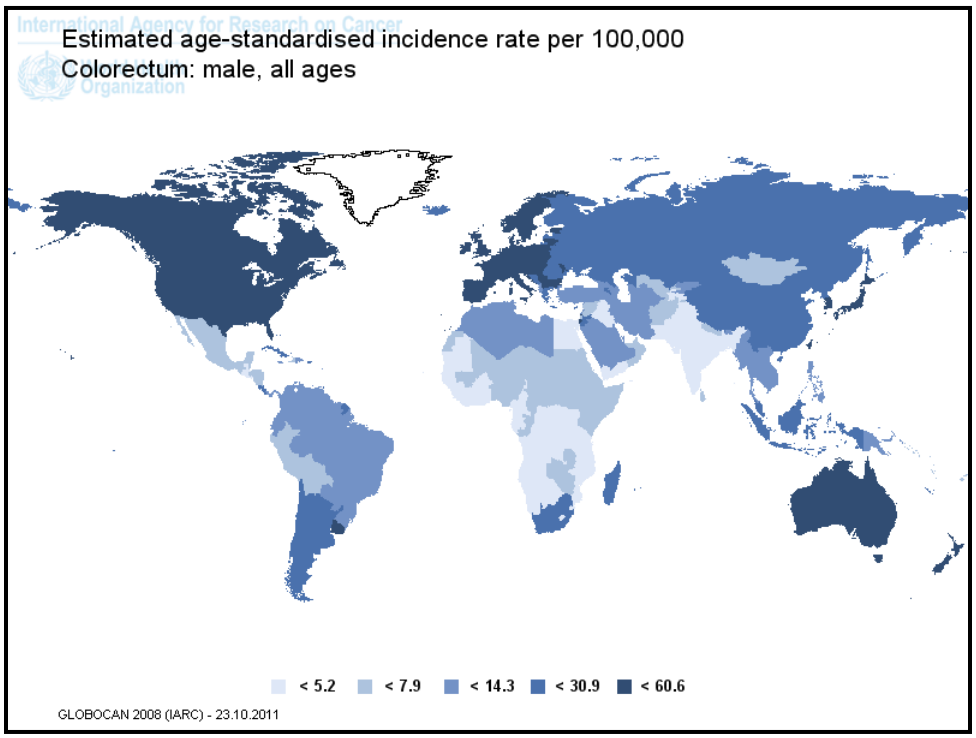

Figure 6a. International Variation in Age-Standarized Colorectal Cancer Incidence Rates Among Males (Globocan 2008).

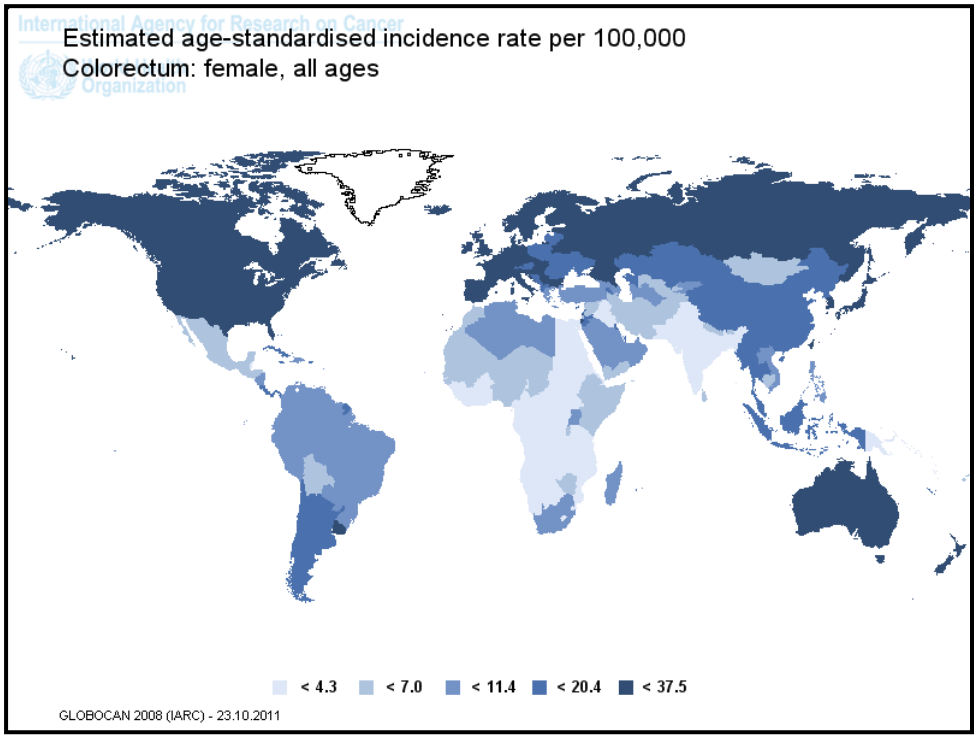

Figure 6b. International Variation in Age-Standarized Colorectal Cancer Incidence Rates Among females (Globocan 2008).

CRC incidence shows important geographic variations and, as it is possible to observe, highest incident rates are seen in North America, Europe, New Zealand, Oceania and recently Japan.

Commonly CRC is diagnosed after 50 – 55 years old and cases that appear before 40's are very rare, except for the hereditary syndromes in which tumors could appear even earlier, at 10 – 15 years (Mobley *et al.* 2009) (Figure 7).

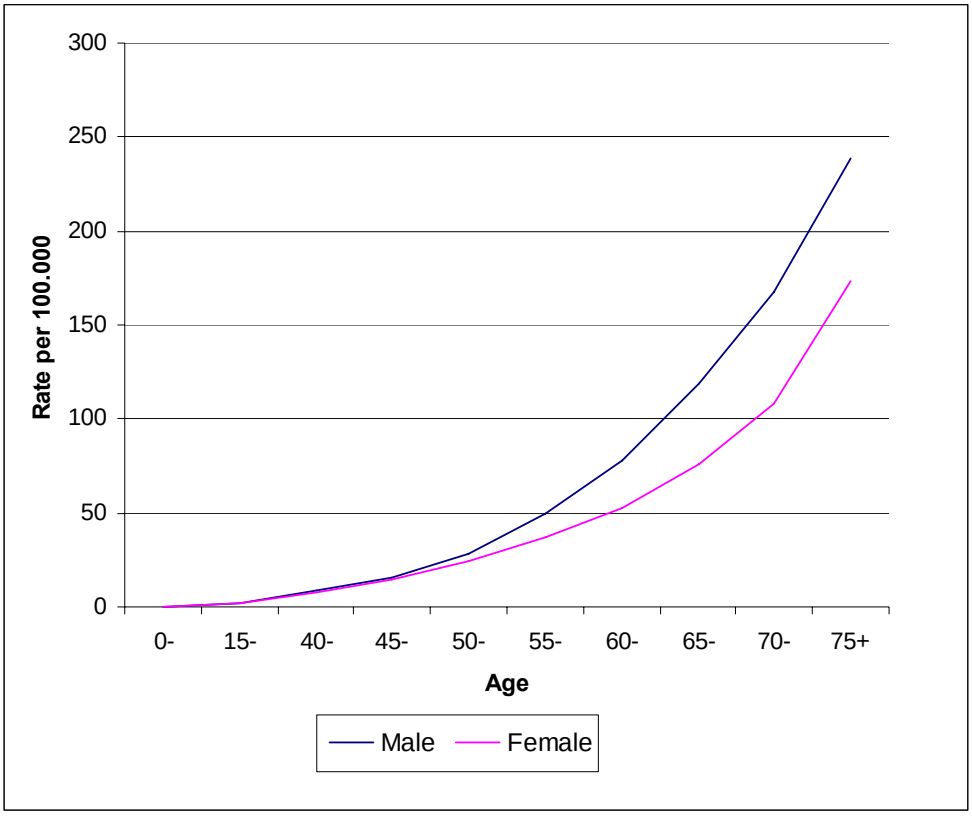

Figure 7. Incidence of colorectal cancer by age and sex (Globocan 2008).

About 75% of CRC cases are defined as sporadic and originate from somatic mutations followed by clone develop in the tumor area. The remaining cases could be explained as consequence of several pathologies like Familial Adenomatous Polyposis, Gardner and Turcot syndrome (1%); Lynch syndrome (4 – 7%); familiar history or adenomatous polyp (15 – 20%); chronic inflammatory intestinal diseases or previous

diagnosis of cancer like ovarian, endometrial, breast, pancreas or stomach (1%) (WHO 2000)

According to the World Health Organization, different classification systems are been used for CRC tumor staging. Originally there was the Dukes classification system, which placed patients into one of three categories (Stages A, B, C). This system was subsequently modified by Astler-Coller to include a fourth stage (Stage D); Gunderson & Sosin subsequently modified it again in 1978. More recently, the American Joint Committee on Cancer (AJCC) has introduced the TNM staging system, which places patients into one of four stages (Stage I–IV).

Table 1. TNM (Tumor, Node, Metastasis) Staging System for Colorectal Cancer

Tumor
T1: Tumor invades submucosa.
T2: Tumor invades muscularis propria.
T3: Tumor invades through the muscularis propria into the subserosa, or into the pericolic or perirectal tissues.
T4: Tumor directly invades other organs or structures, and/or perforates.
Node
N0: No regional lymph node metastasis.
N1: Metastasis in 1 to 3 regional lymph nodes.
N2: Metastasis in 4 or more regional lymph nodes.
Metastasis
M0: No distant metastasis.
M1: Distant metastasis present.
Colorectal Cancer Stage Groupings
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.

1.2.2 Colorectal Polyps

It is well accepted that sporadic CRC cases are preceded by a pre-cancerous lesion that could be described as small evagination of the intestinal epithelium due to cellular hyper-proliferation. These polyps can be sessile or pedunculated, and some could eventually become malignant (Table 2). Indeed, only adenomatous ones are considered pre-cancerous lesions and close to 25% develop into a malignant tumor. As tumor develops, due genetic mutations, specific tissue characteristic are lost, leading to significant changes in the intestinal mucosa allowing establishment of the cancerous lesion or adenocarcinoma (Figure 8).

Table 2. Classification of Colorectal Polyps (Colucci *et al.* 2003)

Histological Classification	**Polyp Type**	**Malignant Potential**
Non-neoplastic	Hyperplastic polyps Hamartomas Lymphoid aggregates Inflammatory polyps	No
Neoplastic (adenomas)	Tubular adenomas (0–25% villous tissue) Tubulovillous adenomas (25–75% villous tissue) Villous adenoma (75–100% villous tissue)	Yes

1.2.3 Familial Adenomatous Polyposis

It is an autosomal dominant hereditary genetic syndrome caused by mutation in the Adenomatous Polyposis Coli (APC) gene, with the consequent appearing of hundreds of adenomatous polyps in the colon rectum. Commonly, these polyps evolve to adenocarcinoma about the third decade of patient life (Figure 8). Even when the main signs are the polyps (over 100), other signs like mandibular osteoma, congenital hypertrophy of the retinal pigment epithelium, dermoid cysts could be present (WHO 2000).

1.2.4 Hereditary Non Polyposis Colorectal Cancer

It is also an autosomal dominant hereditary syndrome caused by mutations of Mismatch Repair genes like MSH2, MLH1, PMS2, MSH6 and MLH3, with MLH1 and MSH2 as the most frequently involved genes. Alterations of these genes induce increased replication errors leading to microsatellite instability (Huang *et al.* 1996, WHO 2000, Yi *et al.* 2011).

1.2.5 Familiar Colorectal Cancer

It includes cases with a previous familiar report of CRC, but which cannot be defined as hereditary syndrome, like FAP or HNPCC. It can be classified as simple or complex, depending on the age and number of relatives with diagnosis of CRC (WHO 2000).

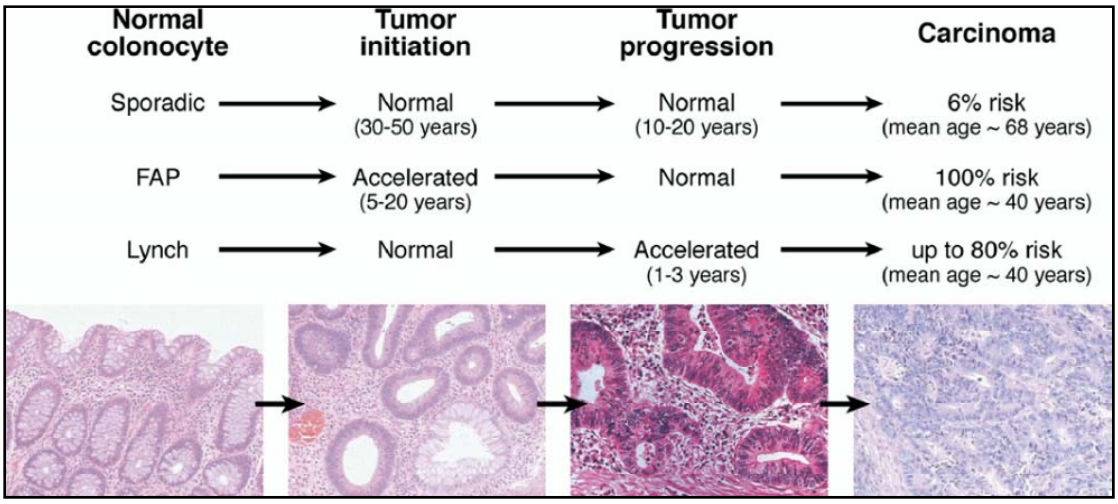

Figure 8. Colorectal cancer progression, from normal epithelium to adenocarcinoma (Grady and Carethers 2008)

1.2.6 Colorectal Cancer Risk Factors

Risk factors related with CRC consider age, lifestyle and genetics. In developed countries, more than 90% of cases are diagnosed in individuals older than 50 years and modifiable lifestyle factors like high alcohol consumption, smoking, physical inactivity, obesity and diet are associated with an increase risk of CRC (Riccardiello *et al.* 2003, Benassi *et al.* 2007, Habermann *et al.* 2009, Garcia *et al.* 2010).

Due to the role played by colon and rectum, establish a correlation between diet and CRC is extremely important and even when results don't always agree, it has been suggested that a diet high in red or processed meat and an inadequate intake of fruits and vegetables increases the risk of CRC (IARC 2002, Riccardiello *et al.* 2003, Garcia *et al.* 2007, World Cancer Research Fund 2007, Habermann *et al.* 2009). Alcohol intake it also been correlated with increased risk of CRC, but the mechanism of action is still unknown (Boffetta and Hashibe 2006).

Recently, it has been proposed that intense physical activity has a protective effect only against colon cancer, having no effect on rectum cancer (Spence *et al.* 2009). However, several studies conducted by the IARC and other research centers failed to

provide a strong correlation between physical activity and CRC, and benefit obtained from it supposed to lie mainly on the ability to increase the intestinal transit, with the consequent reduction of exposition time of intestinal mucosa to potential carcinogens present in the intestinal content (World Cancer Research Fund 2007).

1.2.6.1 *Genetic Instability*

Genetic instability plays an essential role in the development and progression of CRC (Lengauer 1998, Karaman *et al.* 2008). CRC develops through four clinical stages (dysplastic crypts, small benign tumors, malignant tumors invading surrounding tissues, and metastatic cancer), from a benign small lesion (adenoma) to a malignant tumor (carcinoma) through continuous accumulation of genetic and epigenetic alterations, such as inactivation of tumor suppressor genes and activation of oncogenes, which induce and facilitate uncontrollable cell growth, and they are commonly present in advances stages of CRC regardless the possible origin of the pathology (FAP, Lynch syndrome, sporadic) (Powell *et al.* 1992, Carethers 1996, Huang *et al.* 1996, WHO 2000, Grady and Markowitz 2002, Riccardiello *et al.* 2003, Karaman *et al.* 2008, Bousserouel *et al.* 2010).

Two major types of genetic instability have been described for CRC: chromosomal instability and microsatellite instability (Lengauer 1998, Karaman *et al.* 2008). Base substitution/deletion/insertion, chromosome rearrangement, genic amplification and aneuploidy/polyploidy are the most common forms of chromosomal instability (Lengauer *et al.* 1998, Aguilera and Gomez-Gonzales 2008). About 85% of CRCs develop through this pathway, which is characterized by aneuploidy as well as loss of heterozygosity (Karaman *et al.* 2008). Mutations of the APC gene are considered the earliest and the most prevalent genetic change in colorectal tumorigenesis able to facilitate aneuploidy (Jallepalli and Lengauer 2001, Rajagopalen *et al.* 2003, Sieber *et al.* 2003).

On the other hand, microsatellite instability emerges when a lower activity of mismatch repair system verifies. Microsatellites, or Simple Sequences Repeats (SSRs), length result altered in tumor cells respect to normal cells of the same patient.

Additionally, the DNA double-strand breaks, due to the action of intrinsic or extrinsic mutagens, are considered the most critical DNA lesions (Mills *et al.* 2003, Karaman *et al.* 2008), and it has been suggested that defects in the cellular response to double-strand breaks can lead to genetic alteration, chromosomal instability, and ultimately malignant transformation as is possible to see in CRC (Mills *et al.* 2003, Karaman *et al.* 2008).

1.2.7 Colorectal Cancer Screening and Diagnosis

The detection of CRC, at early stages, is one of the proven strategies resulting in a higher cure rate (Rutter *et al.* 2006, Mobley *et al.* 2009). Although CRC is one in the small group of cancers which can be prevented by screening, only 39% of cases are diagnosed at early stages (IARC 2008, Mobley *et al.* 2009).

Subjects can be divided as at generic, intermediate or high risk, considering age (over 50 years old), presence of colon adenomas, relatives with previous diagnose of CRC, chronic intestinal diseases, hereditary syndromes or diagnose of other kind of cancers. Several studies highlight screening advantages for subjects at generic risk of CRC regarding not only the early detection of a treatable lesion, but also the reduction of mortality (Levin *et al.* 2008, Whitlock *et al.* 2008) The current adopted CRC screening procedures include fecal occult blood test (FOBT) and colonoscopy.

It is known that adenomatous polyps may bleed from the very early stages (IARC 2002), but even when this condition was present the amount of blood could be so small or intermittent, that goes unnoticed to naked eye, while adenomatous polyp continues to develop into malignant tumor. This bleeding can be detected through FOBT, who has proven to have a sensibility ranged between 60 to 85% and when positive it indicates three to four fold increased CRC risk compared with negative one. Normally it is recommended to perform FOBT at least every two years and several studies suggest a reduction in mortality rate close to 30% (Hardcastle *et al.* 1996).

The colonoscopy, as a validation test or as screening tool by itself, allows to confirms CRC condition with a 90% sensibility and consents full colon rectum inspection (Schoenfeld *et al.* 2005, Regula *et al.* 2006). Additionally, through colonoscopy it is also possible to remove pre cancerous lesions, reducing CRC incidence and mortality. However, due to the invasive character and discomfort associated with colonoscopy, patients generally reject the test (Bousserouel *et al.* 2010).

Other test like X-ray study, sigmoidoscopy or colonography are well described and used as screening test, however FOB and colonoscopy offers slight advantage over these (Lieberman *et al.* 2000, Johnson *et al.* 2008).

In recent years, several studies have appeared identifying potential cancer markers in serum, plasma and stool in an attempt to improve actual screening procedures (Gupta *et al.* 2008, Levin *et al.* 2008, deVos *et al.* 2009).

1.3 Molecular Epidemiology

The classic epidemiological approach trough retrospective and prospective studies allow to identify and establish possible relations between potential risk factors and a particular disease. However, the critical changes that take place after the interaction between organism and risk factors remain unknown (Legator and Au 1994, Bolognesi *et al.* 2005, Bonassi *et al.* 2007). In the carcinogenic process, early critical changes are of great importance due the fact that they could indicate the establishment and beginning of the process (Legator and Au 1994, Bolognesi *et al.* 2005, Fenech 2008).

The improvement of molecular biology techniques and more accurate scientific instruments have allowed the development of molecular epidemiology. As a discipline, it integrates epidemiologic approaches and merges with molecular biology tools in an attempt to response unsolved questions opened by traditional epidemiology.

The objective of molecular epidemiology is to identify and study biomarkers able to reflect cellular, molecular and genetic changes that take place at early stages of different chronic degenerative diseases. Then, a biomarker can be defined as a molecular or biological measurable event able to highlight critical events potentially dangerous for the organism.

Biomarkers can be classified into three main categories: markers of exposure; markers of effect, and markers of susceptibility (Bolognesi *et al.* 2005).The difference among them resides in the target that look for, while markers of exposure are centered in the risk factor (e.g. a xenobiotic), markers of effect are centered in the biological effect, at molecular level (Legator and Au 1994, Bolognesi *et al.* 2005). On the other hand, markers of susceptibility are centered in the genetic characteristics of the biological response of an organism faced with a certain risk factor (Legator and Au 1994). A brief class description is presented in Table 3.

Table 3. Biomarkers classification

BIOMARKER CLASS	DESCRIPTION
Exposure	They can be the xenobiotic itself or a metabolite, or a product of xenobiotic interaction, or any other event correlated with exposure. They can be divided in two subcategories: internal dose and biological effective dose.
Effect	Any kind of marker able to reflect exposure effect over the whole organism or a particular organ system. They are considered as preclinical indicators and can be specific or nonspecific.
Susceptibility	Includes markers indicative of individual sensitivity to effects of a particular xenobiotic. They can be inherited or induced.

1.3.1 Micronuclei Frequency Analysis

As previously mentioned, genomic instability and/or changes in chromosome number has been described as mainly events correlated with cancer etiopathogenesis (Legator and Au 1994, Gollin 2005), thus the study of genetic damage biomarkers able to highlight this alterations, in different surrogate tissues, are of great interest due to the potential application in prevention and public health.

Micronuclei (MN) (Figure 9) are an early effect biomarker that indicates genome damage at cellular level and consist in small fragments of DNA which during mitosis remain out of main nucleus constituting little nucleus-like forms (Fenech 2008). The events related with MN formation consider chromosome breakage or chromosome malsegregation, which could be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation process (Lengauer *et al.* 1998, Bonassi *et al.* 2007, Karaman *et al.* 2008).

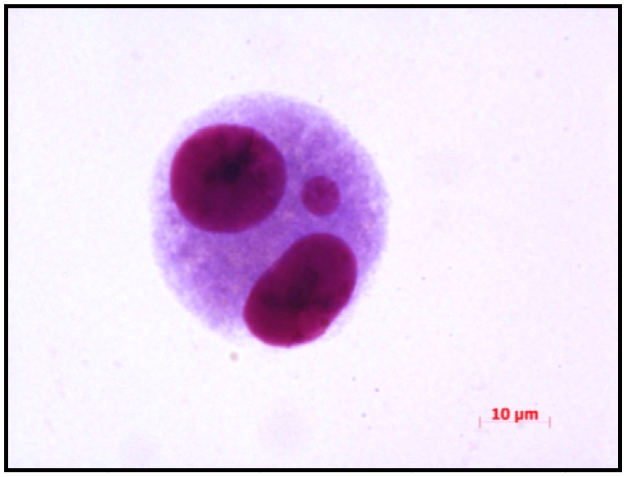

Figure 9. Binucleated lymphocyte.

Micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage (Norppa 2004, Iamarcovai *et al.* 2006, Fenech 2007 and 2008) and has been widely used in molecular epidemiology and cytogenetic to assess presence and extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile (Benassi *et al.* 2007, Bonassi *et al.* 2007, Karaman *et al.* 2008). The measurement of MN frequency in peripheral blood lymphocytes (PBL) has been also successfully used to identify dietary and genetic factors which have a significant impact on genome stability (Benassi *et al.* 2007, Bonassi *et al.* 2007) and it's utility as a prediction cancer risk tool is well documented (Legator and Au 1994, Norppa 2004, Bolognesi *et al.* 2005, Iamarcovai *et al.* 2006, Benassi *et al.* 2007, Bonassi *et al.* 2007).

1.3.2 Clastogenic Factors Analysis

Clastogenic factors (CF) are chromosome-damaging molecules, present in plasma. Several authors have linked oxidative stress induced damage with superoxide anion radical production and have pointed that the latter acts as an initiator for several reactions leading to the formation of clastogenic compounds such aldehyde 4-hydroxynonenal, tumor necrosis factor alpha (TNF-α) and inosine di-and triphosphate (Auclair *et al.* 1990, Emerit 2007, Lindholm *et al.* 2010). Several studies conducted mainly in patients exposed to high

levels of radiation, like Chernobyl disaster workers and child's at present days affected by radiation related diseases, or patients that have received radiotherapy, have also indicated that the MN assay is able to reveal clastogenic activity (Emerit *et al.* 1997, Ballardin *et al.* 2002, Ballardin *et al.* 2004, Lindholm *et al.* 2010). Emerit (2007) suggests that it's possible to observe clastogenic factors (i.e. chromosome damaging) activity in circulating plasma of subjects affected by different kinds of diseases associated with oxidative stress, including radiation exposure, chronic inflammatory diseases, viral infections, ischemia reperfusion injury and hereditary chromosomal instability syndromes

1.3.3 Epigenetic Analysis

Epigenetic (from the Greek, epi-: *επί*- over, above; and -genetics) corresponds to the study of heritable changes in gene expression or in the cellular phenotype caused by mechanisms other than changes in the DNA sequence (Feinberg and Tycko 2009). Cellular differentiation is a well know example of relevance of epigenetic mechanism. If all cells within an organism have the same DNA (Nestler 2009) then the ability to have different cells with different functions must be due to a selective activation or silencing of particular genes within the genome (Grewal 2003). Actually, it has been demonstrated that epigenetic events, altogether with genetic events, play a crucial role in tumor progression (Jordà and Peinado 2010).

Three epigenetic mechanisms are considered the most important ones: genomic imprinting, histone modifications and DNA methylation (Feinberg and Tycko 2004). Genomic imprinting refers to the relative silencing of one parental allele compared with the other parental allele as the consequence of differentially methylated regions within or near imprinted genes. Histone modifications, principally acetylation, methylation and phosphorylation, are important in the transcriptional regulation, due the ability to induce chromatin structure modification, altering DNA accessibility (Feinberg and Tycko 2004). DNA methylation is the most common epigenetic mechanism (Jordà and Peinado 2010) and consists in a covalent modification of DNA (Figure 10), in which a methyl group is transferred from S-adenosylmethionine to the C-5 position of cytosine by a family of

cytosine DNA-5-methyltransferases (Feinberg and Tycko 2004) and occurs predominantly in the cytosines that precede guanines (CpG) (Bird 1986, Deaton and Bird 2011).

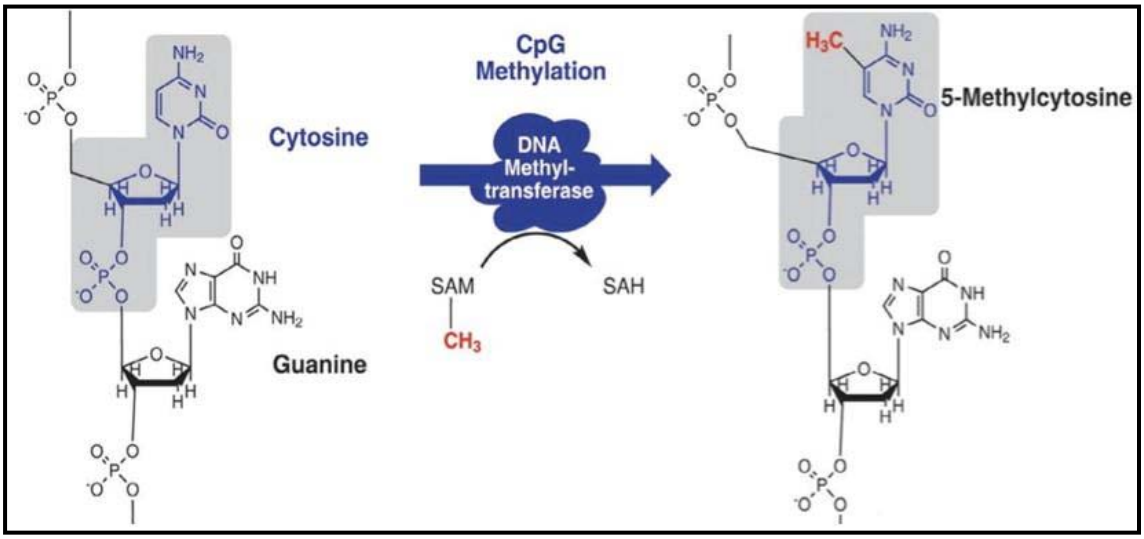

Figure 10. Cytosine methylation.

1.3.3.1 *DNA Methylation*

Even when most human genome lacks of CpG's, there are short genomic regions (500 bp to a few kb) located in the proximal promoter region of approximately 75% of human genes rich in CpG nucleotides, called CpG islands (Jordà and Peinado 2010). Most of this CpG islands are unmethylated and remains unmethylated at all stages of the development and in all tissue types. However a small proportion becomes methylated during normal physiological processes and when it happens the associated gene is stably silent (Jordà and Peinado 2010, Terry *et al.* 2011). Methylation regulates gene expression not only by altering the interaction of transcription factors to DNA, but also through the modification of chromatin conformation (Nan *et al.* 1998, Jordà and Peinado 2010). In recent years, the study of DNA methylation and its role in tumorigenesis has become one of the main issues in molecular oncology (Jordà and Peinado 2010, Deaton and Bird 2011).

Three strategies are commonly used to approach DNA methylation studies, digestion of DNA with methylation sensitive or insensitive restriction enzymes, the chemical modification of DNA by bisulphite, and the purification of the methylated fraction of the genome using antibodies (Jordà and Peinado 2010). Bisulphite-modified DNA based studies are the method of choice in most laboratories (Feinberg and Tycko 2004, Jordà and Peinado 2010, Deaton and Bird 2011) and Polymerase Chain Reaction (PCR) analysis, specially through methylation-specific PCR (MSP), is the most widely used method to detect CpG-island methylation status (Jordà and Peinado 2010).

However, under standard PCR conditions, the epigenetic information, as methylation pattern, is generally lost because DNA polymerase does not distinguish between methylated and non-methylated cytosine, so the polymerase adds a guanine and then a non-methylated cytosine in both situations. As a consequence, each originally methylated allele is diluted to a concentration impossible to analyze, so the DNA must be modified in a way that allows methylated information to remain preserved. Treatment with sodium bisulphite, which converts non methylated cytosines into uracil (Clark *et al.* 1994), is used to avoid this problem.

The protocol described by Frommer and colleagues (Frommer *et al.* 1992, Clark *et al.* 2006) has been widely used for the treatment with sodium bisulphite and when is conducted under appropriate conditions, the expected conversion level of unmethylated cytosines is about 99% (Clark *et al.* 1994). Due the fact that the degree of deamination of 5'-methylcytosine to thymine is much slower than the conversion of cytosine to uracil, it is assumed that the only remaining cytosine after treatment with sodium bisulphite are those derived from 5'-methylcytosine. During the subsequent PCR, the uracil residues are transcribed as Thymine. The procedure is based on the chemical reaction of single-stranded DNA with sodium bisulphite (HSO3-) at low pH and high temperatures. The chemical reaction of each step is as follows: cytosine carbon-6 sulphonation, irreversible hydrolytic deamination of carbon 4 that produces a sulphonate uracil, and finally the following desulphonation under alkaline conditions to generate uracil. Methylation of carbon-5 prevents the carbon-6 sulphonation in the first step of reaction. Although the 5-methylcytosine can react with sodium bisulphite, this reaction is extremely slow, and the balance favors the 5'-methylcytosine rather than thymine (the deamination product of 5'-methylcytosine). In conclusion, the treatment with sodium bisulphite converts unmethylated cytosine of the original strand of DNA to uracil, while methylated cytosines remain cytosines (Frommer *et al.* 1992, Clark *et al.* 1994 and 2006).

1.3.3.2 *DNA Methylation and Colorectal Cancer*

Colorectal Cancer is a well established model for DNA methylation (Carmona and Esteller 2011). Using different approaches, a methylation pattern has been identified for several genes correlated with CRC (Esteller 2007, Jordà and Peinado 2010).

Table 4. Methylation studies of genes correlated with CRC

Ogino S. *et al.* 2007	CACNA1 G	voltage-dependent calcium chanel alpha 1
	CDKN2A	cyclin-dependent kinase inhibitor 2A
	CRABP1	cellular retinoic acid binding protein 1
	IGF2	insuline growth factor 2
	MLH1	mutL homolog 1
	NEUROG1	neurogenin 1
	RUNX3	runt-related transcriptor factor 3
	SOCS1	suppressor of cytokine signaling 1
Lofton-Day *et al.* 2008	TMEFF2	tomoregulin 2
	NGFR	nerve growth factor receptor
	SPET9	septin 9
Lee K. -H *et al.* 2011	hMSH2	mutS homolog 2
	MGMT	methyl-guanin DNA methyl transferase
	MLH1	mutL homolog 1

Results obtained through these and other studies have detected possible methylation patterns correlated with chromosome instability (CIN) and microsatellite instability (MSI), previously mentioned as the main genetic pathways that lead to CRC, but have also identified a new possible genetic variation named CpG island methylator phenotype (CIMP), suggesting a distinct clinicopathological feature with a particular methylation pattern (Silver *et al.* 2011, Zlobec *et al.* 2011). Additionally, some studies indicate possible methylation variations for cancer recurrence risk correlated not only to CRC specific genes but also with extra cellular matrix (ECM) pathways (Carmona and Esteller 2011, Yi *et al.* 2011), opening up additional areas of research.

Although the regulation of gene expression by aberrant methylation has been well characterized in tumor biology in general and extensively described for CRC (Lofton-Day *et al.* 2008), the vast majority of available studies and literature is referred to tissue-specific methylation (Terry *et al.* 2011). Only in recent years, in an attempt to improve actual CRC screening procedures, studies have appeared identifying potential DNA methylation-based biomarkers in serum, plasma and stool (deVos *et al.* 2009, Shivapurkar and Gazdar 2010, Ahlquist *et al.* 2011).

One of the latest DNA methylation-based biomarker for CRC is the plasmatic SEPT9 (septin 9 gene) promoter hypermethylation analysis but, even when presented positive results in different studies (Lofton-Day *et al.* 2008, deVos *et al.* 2009, Tänzer *et al.* 2010), some concerns about sensitivity still remain and it has been suggested the stool SEPT9 promoter hypermethylaton analysis to minimize this problem (Ahlquist *et al.* 2011). Recently, NEUROG1 and NOTCH 3 have been suggested also as a possible DNA methylation-based biomarker (Miyamoto and Rosenberg 2011, Serafin *et al.* 2011). However, further studies must be conducted to validate these biomarkers, as well as to find additional ones that helps not only to detect but also to predict disease outcome.

1.3.3.3 *Septin 9 (SEPT9)*

Septins are a gene family of cytoskeleton related proteins, encodes cytoplasmic GTP binding and hydrolyzing proteins, consisting of 14 members that associate and interact with actin and tubulin. They are involved in diverse cellular processes including cytokinesis, vesicle trafficking, apoptosis and maintenance of cell polarity (Scott *et al.* 2005, Connolly *et al.* 2011).

The mechanistic basis for the wide broad of septins functions remains obscure and even when most of data about biology and chemistry comes from different organism models, particularly in yeast, several lines of evidence suggest a role for septins in the development of neoplasia (Scott *et al.* 2005, Connolly *et al.* 2011, Peterson *et al.* 2011). Indeed, current available evidence shows a deregulated activity of septins (Scott *et al.* 2005).

In particular, SEPT9 gene has very unique characteristics and has been shown to act as an oncogene although tumor suppressor functions have been reported (Table 5) (Connolly *et al.* 2011). In fact, overexpression of SEPT9 has been reported in diverse human tumors including breast, central nervous system, endometrium, kidney, liver, lung, lymphoid, esophagus, ovary, pancreas, skin, soft tissue and thyroid (Scott *et al.* 2005). On

the other hand, SEPT9 silencing has been reported in colorectal and head and neck cancer (Lofton-Day *et al.* 2008, deVos *et al.* 2009, Tänzer *et al.* 2010, Ahlquist *et al.* 2011).

Table 5. SEPT9 link to cancer (extracted from Connolly *et al.* 2011)

Gene	Oncogenic function	Tumor supressor function	Tumor type
SEPT9	Amplification / Overexpression		Breast
	Upregulation		Ovary
	Gene fusion		AML, ALL
		Hypermethylation	Colon, Head and Neck
		Deletion	Ovary, Breast, Hodking lymphoma

AML = acute myeloid leukemia, ALL = acute lymphoblastic leukemia

1.3.3.4 *NOTCH3*

Notch genes encode transmembrane receptors (Notch1, 2, 3 and 4) involved in the NOTCH signaling pathway (Figure 11), which deregulation has been observed in different kind of tumors (Serafin *et al.* 2011) playing a critical role in maintaining cancer stem cell properties (Miyamoto and Rosenberg 2011). The Notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism essential for proper embryonic development, due to its demonstrated role in cell differentiation and proliferation (Miyamoto and Rosenberg 2011, Serafin *et al.* 2011).

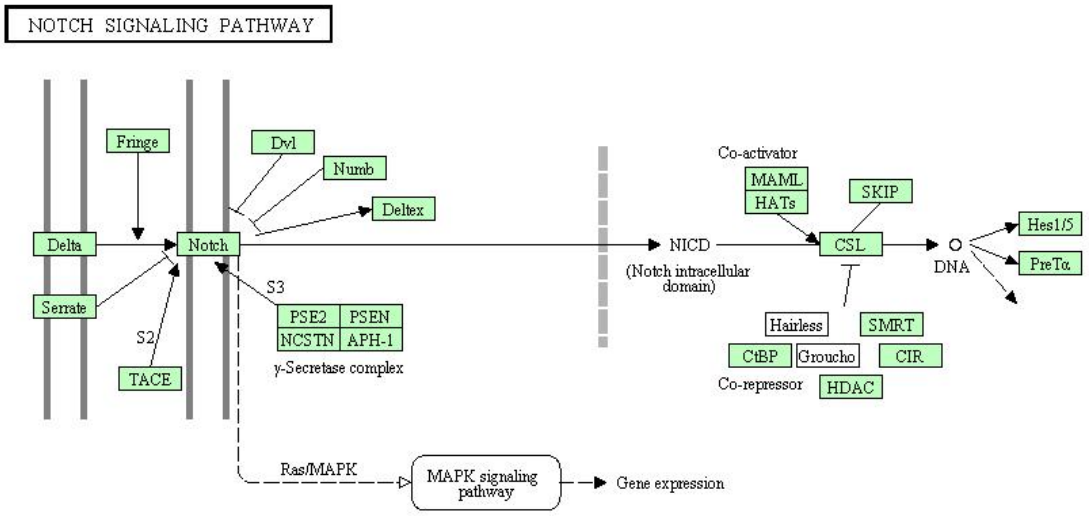

Figure 11. Overview of NOTCH signaling pathway. Notch proteins are single-pass receptors with an intracellular domain (NICD). When activated, the NICD translocates to the nucleus where interact with different transcription factors leading to activation of Notch target genes (source Kyoto Encyclopedia of Genes and Genomes).

Recent studies have linked Notch1 with Wnt pathway in colon adenomas, and with Jagged-1 and DLL ligands expression in CRC (Serafin *et al.* 2011). However, due the importance and complexity of NOTCH pathway, due to the possible cross-talk with additional signaling pathways such Wnt, the information available about involvement of other Notch receptors in CRC remains insufficient (Miyamoto and Rosenberg 2011, Serafin *et al.* 2011).

2. AIM OF THE STUDY

Main efforts have been made to increase screening rates among population at risk for CRC but due the poor patient compliance to current available screening tests, the identification and development of new markers obtained from easily accessible tissues, leading to a less invasive screening test, have become of great importance (deVos *et al*. 2009). Several studies have appeared identifying potential cancer markers in serum, plasma and stool in an attempt to improve actual screening procedures (Gupta *et al.* 2008, Levin *et al.* 2008, deVos *et al.* 2009). Additionally, continuous improvements of molecular techniques have allowed increasing knowledge regarding epigenetic mechanisms and carcinogenic process, and one of the latest DNA methylation-based biomarker for CRC is the plasmatic SEPT9 promoter hypermethylation analysis (Lofton-Day *et al.* 2008, deVos *et al.* 2009, Tänzer *et al.* 2010). However, other genes such NOTCH3 and NEUROG1, have been also proposed as potential biomarkers for CRC (Miyamoto and Rosenberg 2011, Serafin *et al.* 2011). Even when great steps have been done to understand CRC, further studies are still needed in order to really improve screening and early detection rates (Lofton-Day *et al.* 2008, deVos *et al.* 2009, Tänzer *et al.* 2010, Ahlquist *et al.* 2011). Thus, the general objective proposed for the research project has been the identification and evaluation of biological effect biomarkers in haematic tissue related with CRC risk.

In order to achieve this goal, specific objectives have been established:

- To evaluate Micronuclei (MN) frequency in peripheral blood lymphocytes from subjects resulted positive to fecal occult blood test (FOBT) and examined by colonoscopy.

- To evaluate plasma ultrafiltrate capacity, of subjects resulted positive to fecal occult blood test (FBT) and examined by colonoscopy, to induce MN formation (CF-MN) in healthy donor peripheral blood lymphocytes.

- To evaluate epigenetic modifications correlated with histopathological changes of colon-rectum. Particularly, SEPT9 and NOTCH3 promoter methylation profile in subjects resulted positive to fecal occult blood test (FOBT) and examined by colonoscopy.

3. MATERIALS AND METHODS

In order to identify molecular biomarkers correlated with CRC risk, a molecular epidemiologic study was conducted. Main steps are indicated below.

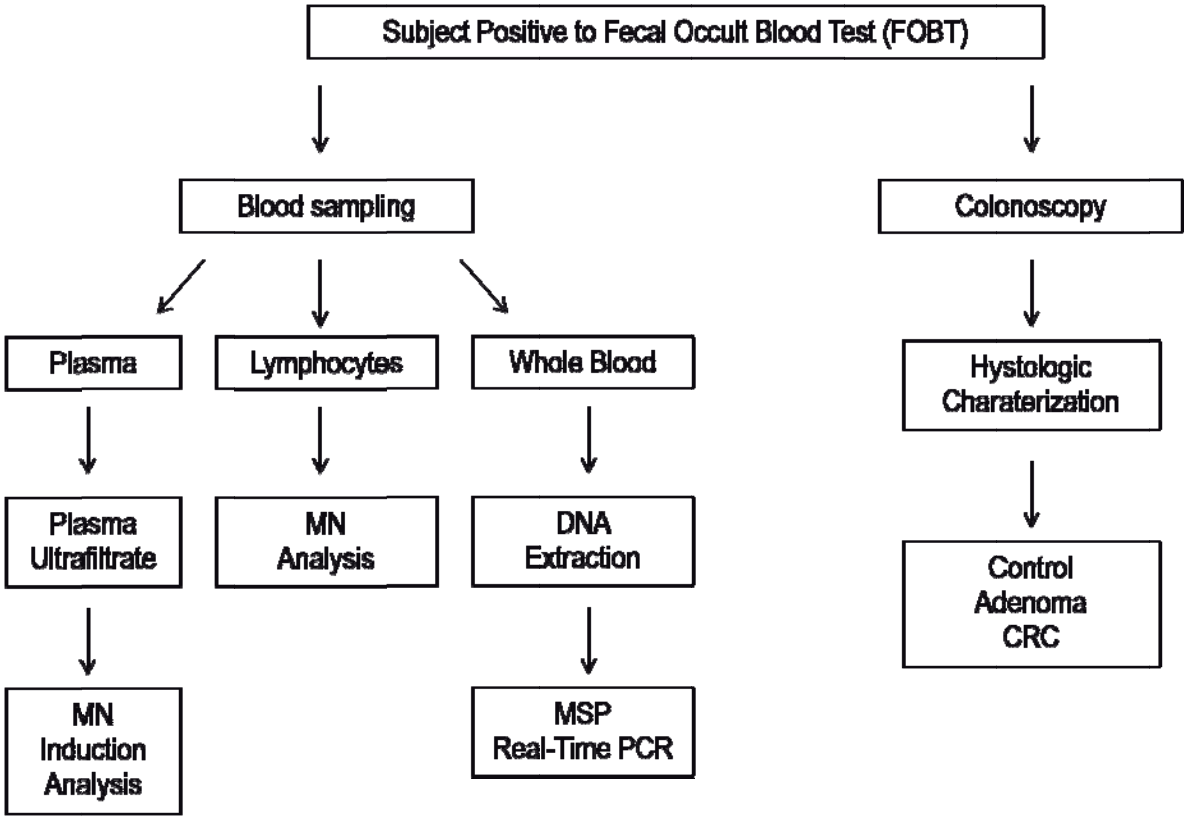

Figure 12. Study workflow. MN = micronuclei, MSP = methylation specific PCR, PCR = polymerase chain reaction, CRC = colorectal cancer.

3.1 Study Population

All subjects for the study were randomly enrolled among the voluntary participants of a CRC screening program organized by specialized physician from the Department of Clinical Medicine of the Sant'Orsola Malpighi Hospital–University of Bologna. Study population comprise 75 subjects aged between 50-80 years, resulted positive to FOBT and classified by histological lesion at the baseline colonoscopy in three groups: a) Control: normal epithelium (26 subjects, 7 females and 19 males); b) Adenoma: adenoma (23

subjects, 10 females and 13 males); and c) Carcinoma: CRC (26 subjects, 10 females and 16 males). All the subjects were informed about the study aim according to WMA Declaration of Helsinki (World Medical Association 2008) and have provided informed consent. Data regarding demographic characteristic, gastrointestinal history, occupational history, diet and lifestyle habits have been collected. Body Mass Index (BMI) has been included as biological parameter linked to body fatness.

3.2 Blood Sample

Peripheral blood samples were collected by venipuncture from subjects before colonoscopy by medical workers into heparinized tubes and properly coded. Within 1 hour of collection, blood samples were centrifuged at 1500 g, for 15 min at 4 °C to obtain the plasma and buffy-coat from each sample. Processing and scoring of the samples were performed blind and concurrently. At the end of the study, information regarding pathological status and recorded data were linked to the code number, becoming available for statistical analysis.

3.3 Micronuclei Analysis

The MN analysis was performed using the cytokinesis-block assay descrived by Fenech (Fenech and Morley 1985, Fenech 2007). Peripheral lymphocytes were isolated from the buffy-coat of each patient by Histopaque gradient centrifugation (Sigma, St. Louis, MO, USA). Cultures were set up using 2 x 10^6 lymphocytes for each sample in 5 ml RPMI 1640 (Sigma) medium, containing 15% fetal calf serum (Sigma), 1% phytohaemagglutinin (Sigma), 1mM L-glutamine (Sigma), 100 UI penicillin and 100 µg/ml streptomycin (Sigma). Cultures were incubated at 37 °C and 5% CO_2 for 72 h. Cytochalasin-B (Sigma) was added (final concentration 6 µg/ml) for the last 28 h. Cells were collected and treated with a mild hypotonic treatment (one part RPMI 1640 medium and one part distilled water) and fixed with a mixture of methanol/glacial acetic acid. The slides were prepared by cytocentrifugation, air-dried and then stained by conventional May-Grünwald Giemsa staining (Sigma). Cell-cycle parameters were evaluated by the nuclear

division index (NDI). This index was calculated by scoring at least 1000 cells for the presence of one, two, three or more nuclei according the following formula: NDI = $(1M_1 + 2M_2 + 3M_3 + 4M_4)/n$, where $M_1 – M_4$ indicates the number of cells with 1 – 4 nuclei and n indicates the total number of cells scored. MN were scored using a Zeiss Axioplan microscope at 1000X and, in accordance with standard criteria, 2000 binucleated lymphocytes were evaluated for each patient (Fenech 2007).

3.4 Clastogenic Factors (CF) Analysis

An aliquot of plasma from each patient was ultrafiltered at 800 g for 2 hours using Centriplus tubes, with 10 kDa cutoff (Millipore). This procedure removes all high-molecular-weight plasma components that might disturb culture growth because of blood group incompatibilities. In addition, it eliminates cells that might still be present in the plasma after centrifugation. The plasma ultrafiltrate from each patient was immediately frozen at −80 °C until use. CF-MN analysis was also performed using the cytokinesis-block technique. In this case, peripheral lymphocytes were isolated from a buffy-coat of a healthy no-smoker male provided by AVIS (Italian Association of Voluntary Blood donors) using a density gradient (Histopaque, Sigma). Lymphocytes cultures were set up by incubating 2 x 10^6 cells in 5 ml RPMI 1640 (Sigma) medium, containing 15% fetal calf serum (Sigma), 1% phytohaemagglutinin (Sigma), 1mM L-glutamine (Sigma), 100 UI penicillin and 100 µg/ml streptomycin (Sigma), and 100 µl of plasma ultrafiltrate obtained from each subject were added to paired lymphocytes cultures. After 44 h, all cultures were supplemented with cytochalasin-B (final concentration 6 µg/ml). At the end of 72 h incubation, cells were collected and treated with a mild hypotonic treatment (one part RPMI 1640 medium and one part distilled water) and fixed with a mixture of methanol/glacial acetic acid. The slides were prepared by cytocentrifugation, air-dried and then stained by conventional May-Grünwald Giemsa staining (Sigma). CF-MN were scored as mentioned previously.

3.5 Cell Culture

Additionally, CaCo2 cells (Istituto Zooprofilattico della Lombardia e dell'Emilia, Iatly) were cultured in Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) with 20% fetal calf serum (Lonza) and 1mM L-glutamine (Sigma), 100 UI penicillin and 100 µg/ml streptomycin (Sigma). Cultures were done in triplicates. At the end of culture, cells were Trypsinized (0.25% Trypsin-EDTA solution, Sigma), counted and divided in aliquots up to 5 x 10^6 cells.

3.6 DNA extraction and purification

DNA was extracted from 100 µl of whole blood, plasma and CACO2 cells using ZR Genomic DNA (Zymo Research, Irvine, CA, USA) following manufacturer's instructions. Main steps have considered:

1. Protein digestion with Digestion Buffer and Proteinase K.
2. Mix and incubation at 55 °C for 20 min.
3. Addition of 700 µl of Genomic Lysis Buffer and vortex.
4. Transference to an extraction column and centrifuge at 10.000 x g for 1 min.
5. Addition of 200 µl of DNA Pre-Wash Buffer. And centrifuge at 10.000 x g for 1 min.
6. Addition of 400 µl of g-DNA Wash Buffer. And centrifuge at 10.000 x g for 1 min.
7. Addition of 20 µl of DNA Elution Buffer. Incubation at room temperature for 5 min. and centrifuge at top speed for 30 sec.

Sample DNA amount was determined by spectrophotometry at 260 nm and DNA was stored at -20°C until use.

3.7 DNA Bisulphite Conversion

The bisulphite modification of DNA samples previously extracted was carried out with the EZ DNA Methylation Kit (Zymo Research) according to manufacturer's

instruction (Figure 13). For each conversion reaction, 500 ng of DNA or closest (in case of insufficient amount of DNA) was used. Main steps consider:

1. Addition of 5uL of M-Dilution Buffer to DNA sample and addition of Nuclease-Free water up to 50µL volume.
2. Incubation at 37 °C for 15 min.
3. Addition of 100µL of CT Conversion Reagent.
4. Incubation at 50°C for 12 to 16 hours in the dark.
5. Incubation of samples at 4 °C (on ice) for 10 min.
6. Transference of each sample to individual separation columns, containing 400µL of M-Binding Buffer.
7. Centrifuge at top speed for 30 sec.
8. Addition of 100 µl of M-Wash Buffer and centrifuge at top speed for 30 sec.
9. Addition of 200 µl of M-Desulphonation Buffer and incubation at room temperature for 15-20 min, then centrifuge at top speed for 30 sec.
10. Addition of 200 µl of M-Wash Buffer and centrifuge at top speed for 30 sec. to wash the DNA. This step is repeated once.
11. Addition of 20 µL of M-Elution Buffer and centrifuge at top speed for 30 sec. to release DNA from the resin.

Converted DNA is ready to use or stored at -80 °C for up to three months. Reported reaction conversion efficiency and DNA recovery is up to 99% and 80%, respectively.

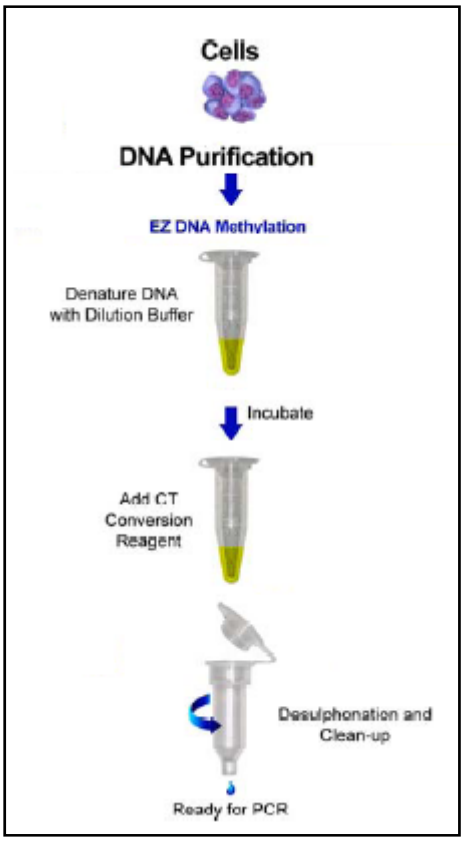

Figure 13. EZ DNA Methylation procedure (source Zymo Research, 2011).

3.8 MSP Primers

In order to analyze SEPT9 and NOTCH3 promoter methylation status, MSP primers were deigned.

SEPT9 and NOTCH3 gene sequence was retrieved from online public databases and CpG islands were detected upstream in the promoter region (Figure 14). Then, several pairs (forward / reverse primers) of suitable sequences were produced due to complementary lost between DNA strands after bisulphite conversion (Table 6).

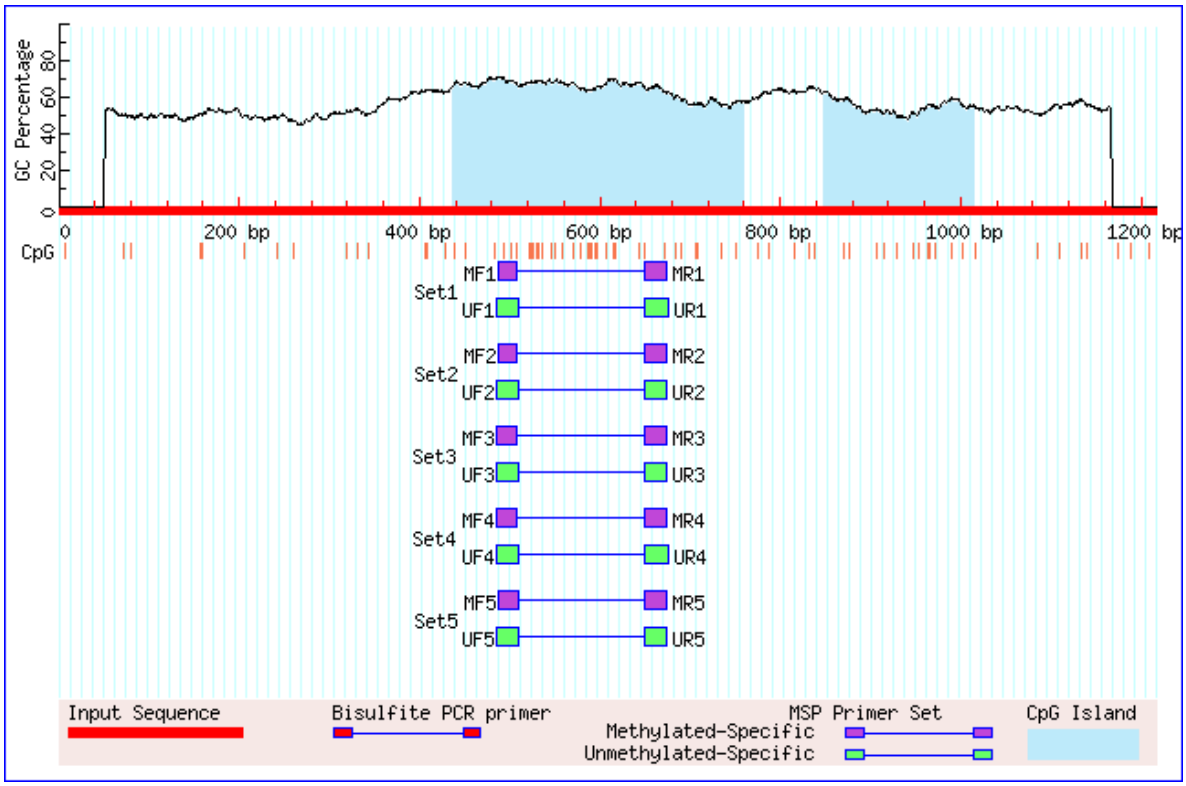

Figure 14. Representation of Promoter CpG Islands and potential MSP primers.

Primers synthesis was requested to CyberGene (CyberGene AB, Stockholm, Sweden).

Table 6. SEPT9 and NOTCH3 MSP primers sequence

Gene	Methylation Status	Direction	Sequence (5' -> 3')
SEPT9	M	F	TTTAAGTTTAAGGAAATCGTAGTATCG
	M	R	AACCACCGAATCTACCTACGAA
	U	F	AATTTTTAAGTTTAAGGAAATTGTAGTATT
	U	R	CAAACCACCAAATCTACCTACAAA
NOTCH3	M	F	TTGTTTCGGTTTTAGAGGTGTTC
	M	R	CTTCGCCGAAATAAAACGAC
	U	F	TTTTGGTTTTAGAGGTGTTTGG
	U	R	CAACTTCACCAAAATAAAACAAC

M/U = methylated/unmethylated; F/R = Forward/Reverse

3.9 rqReal-Time PCR

For relative quantification (rq) PCR, a SYBR Green based PCR kit was used (Maxima SYBR Green/ROX qPCR Master Mix 2x, Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA). Each 25 µl of PCR reaction contains: 1 µl of eluted bisulphite modified DNA (10 ng total DNA), 12.5 µl of SYBR Green PCR Master Mix, 3 µl of F + R primer mix (0.3 µM final concentration), and 8.5 µl of DNase-free water. To normalize for the amount of input DNA, MYOD gene was used as housekeeping. Two replicates for each sample were used.

rqReal-time PCR conditions were 95 °C for 10 minutes followed by 40 cycles of 95 ° C for 15 s, 50 - 65 °C for 30 s, 72 ° C for 60 s with data acquisition after each cycle in a 7900HT Fast Real-Time PCR System (Applied Byosistems, CA, USA). Cycle threshold (Ct) value of each sample was recorded.

Amplification products were verified by melting curve analysis (95 °C for 1 min, 55 °C for 1 min, followed by 80 cycles/10 sec. each of 0.5 °C increasing incubation temperature) with data acquisition after each cycle. For additional verification, the correct length and purity of PCR products were verified by agarose gel electrophoresis (1.5% agarose).

Methylation percentage was calculated according Lehmann and Kreipe method (2004): 1/R + 1 multiplied by 100. Positive control (100% methylated) and standard curves were produced using universally methylated/unmethylated DNA (Qiagen, Hilden, Germany).

3.10 Statistical analysis

One way-ANOVA followed by Bonferroni's multiple comparison test, was used to asses statistically significant differences of: MN frequency between Control, Adenoma and CRC groups; and MN frequency induced by plasma ultrafiltrate of Control, Adenoma and

CRC groups. Given the unknown nature of methylation data, normal distribution was evaluated through Shapiro-Wilk normality test. One-way Anova or Kruskall-wallis test, followed by Bonferroni or Dunn's post hoc-test, were used to assess statistically significant differences of SEPT9 and NOTCH3 promoter methylation percentage among studied groups. MN frequency and induced MN frequency are reported as mean MN ± DS.

To assess the relationship between peripheral blood lymphocyte MN frequency and plasma ultrafiltrate capability to induce MN frequency variations according studied groups, linear regression analysis was used. While multiple linear regression analysis was applied to assess relationship between SEPT9 and NOTCH3 promoter methylation percentage and MN frequency.

Multinomial logistic regression analysis was performed to evaluate the relative risk of different variables over the pathologic status of the study population.

Descriptive statistic, normality test, One-way ANOVA, Kruskall-Wallis, post hoc tests and linear regression analysis were performed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). While multiple linear regression and multinomial logistic regression analysis were carried out using R software (Croissant 2011, R development core team 2011).

4. RESULTS AND DISCUSION

The age and distribution of gender are fundamental to understand the demographic behavior of CRC among population (table 7). The average age considered in the present study (MEAN ± DS; controls: 59.3±6; adenoma: 60.43±6.14; CRC: 67.69±9.76) is in agreement with previous reports of different international cancer organizations, as the American Cancer Society and IARC, about the main age related with CRC development. But, even when all the subjects considered in this study belong to the age risk population, it was possible to observe that CRC group showed a statistically significant higher age not only regarding the control ($p \leq 0.001$) group, but also regarding the adenoma group ($p \leq 0.01$). This information allows confirming the high correlation between age and risk of CRC development often described in literature, and additionally highlights that is possible to observe the same trend also inside the age range considered at high risk. The female/male ratio between study groups indicate that CRC cases are more common among males than females (Table 7), situation that agrees with the same reports of international cancer organizations which suggest that this situation will be explained by lifestyle differences between both sexes, and the possible protection role that may plays hormones as estrogens and progestin (WHO 2000, World Cancer Research Fund 2007). However, the same international agencies have hypothesized that in the future sex could be less determinant as risk factor due sociologic changes that take place today, such as smoking habit or increased women work rate. Indeed, our results show no statistically significant differences between sexes regarding CRC condition (Table 7).

As previously mentioned, in the present study, BMI was included as a biological parameter linked to body fatness and it is important to note that BMI range of study population, according with the "Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults" (National Heart, Lung and Blood Institute 1998), was quite narrow (21 – 29) and has excluded obese subjects (≥ 30), which are describe as a high risk subpopulation. In fact, no statistically significant differences were observed in BMI values among the studied groups. The voluntary condition of the

screening program may explain the low participation of obese patients in this kind of programs.

Figure 15 shows the result of the micronuclei analysis, reported as MN frequency/1000 Binucleated cells. MN frequency was significantly higher in subjects with CRC (17.23 ± 6.3) and adenoma (11.87 ± 3.2) than control (8.3 ± 1.8) ($p \leq 0.001$ and $p \leq 0.01$, respectively). Also, a statistically significant difference in MN frequency was observed between subjects with CRC and adenoma ($p \leq 0.001$). This result seems to support the concept reported by Bonassi *et. al.* (2007) and Karaman *et al.* (2008) that an elevated frequency of MN it is closely related with chromosome damage often observed in carcinogenic process. Furthermore, a wide range of studies indicate that CRC progress through the accumulation of several genetic changes even at the earliest stages of the disease, thus this results suggest that MN frequency varies following CRC dynamics through the different main stages of this pathology (Carethers 1996, Lengauer *et al.* 1998, Karaman *et al.* 2008, Bousserouel *et al.* 2010).

Indeed, the Relative Risk associated with the different variables considered in the study (Table 8), assessed by multinomial logistic regression analysis in a theoretical model which includes MN frequency, age, gender, BMI and smoke habit, indicates that an elevated MN frequency leads to a 71% increased risk to develop adenoma and when associated with an elevated age, it leads to a 113% increased risk for CRC, considering Control group as reference. Several Authors have indicated that age is strongly related with MN fomation (Iamarcovai *et al.* 2006, Bonassi *et al.* 2007, Karaman *et al.* 2008) and seems logic to think that age plays, in the suggested model, not only a role in CRC risk but also in MN formation. To evaluate this situation, the influence of the different variables considered in the study on the MN frequency was assessed by multiple linear regression analysis (Table 9), and even when a positive and statistically significant correlation was found between age and MN, age variation was able to explain only 29% of the variation observed for MN frequency, suggesting underlying mechanisms that lead to an increased MN frequency among adenoma and CRC subjects. Additionally, Fenech *et al.* (2011) have pointed that age plays a significant role in spontaneous MN frequency and described a

monotonically increase in MN frequency over 30 years. However, for the last decades (≥ 50 years) the rate of the increase seems similar and age influence seems to be lower, fact that is consistent with our data.

Regarding the other variables, (gender, BMI and smoke habit) none of them shows influence over pathologic status (Table 8), even when all have been described as risk factor for CRC. As previous mentioned, and as proposed by several international agencies, sociologic changes may have altered the influence of gender and further studies must be conducted to elucidate the real contribution of gender to this particular disease. About BMI, Ning *et al.* (2010) have reported a strong relationship between colon CRC and this variable, and an 18% increased risk each 5 kg/m^2 of BMI gain. On the other hand, smoke habit has been described as one of the most critical factors related with CRC, regrettably, the proportion of never smokers and ex/currently smokers was not ideal and the lack of the latter has influenced the results regarding this variable. It is possible to hypothesize that the voluntary character of the screening program may be negatively influenced obese and ex/current smokers to avoid participate in the program.

About, CF-MN analysis (Figure 16), a statistically significant difference was observed between CRC (8.8 ± 3.5) and control (6.6 ± 1.9) ($p \leq 0.05$). Several authors have pointed the mechanism that lead to spontaneous MN formation (Fenech *et al.* 2011) and Lindholm *et al.* (2010) have suggested that plasma ultrafiltrated from patients affected by different diseases can induce MN formation through clastogenic activity. Our results allow hypothesize the same kind of activity in the plasma ultrafiltrate from CRC subjects. Furthermore, the use of 10 KDa cutoff filter enhance the importance of small molecules in generation and perpetuation of genetic damage. Moreover, as for spontaneous MN frequency, we test the influence of previously studied variables on plasma utrafiltrate capacity to induce MN formation (Table 10) being observed that none of the variables was related with this capacity, suggesting that the same underlying mechanism which lead to spontaneous MN formation are related with plasma constituents being able to induce genetic damage. This situation was later confirmed when relationship between spontaneous MN frequency and CF-MN frequency was tested (Figure 17) and a positive correlation

founded (0.33, $p \leq 0.001$). Traditionally, clastogenic activity has been assessed by chromosome aberration analysis and only in recent years micronuclei has been demonstrated as a reliable indicator (Lindholm *et al.* 2010), however, as previously mentioned, both aneuploidy and clastogenic events can induce micronuclei formation, thus, further analysis, such as *fluorescence in situ hybridization*, must be applied in order to effectively establish the mechanism that lead to an increased micronuclei count (Fenech M. 2011). Even when further studies must be conducted to elucidate if this plasma factors induce genetic damage through clastogenic or aneuplodogenic mechanisms, it's possible to hypothesize that CF should be considered not only as an oxidative stress biomarker (Emerit 2007, Maffei *et al.* 2011), but also as an active agent which could perpetuate and even enhance the genetic damage observed in the carcinogenic process.

Regarding MSP analysis, it was observed that methylation level, in percentage, of SEPT9 promoter region was statistically significant lower in CRC subjects (60.14 ± 23.66) respect controls (79.71 ± 16.24) ($p \leq 0.01$). On the other hand, methylation level in CaCo2 cells, for the same gene, was close to 100% (97.94) (Figure 18). Although these results appear contradictory, they are in agreement with what reported in literature. Scott *et al.* (2005) have observed an overexpression of SEPT9 in different tissues, included the hematic one, in different types of cancer, while at CRC tissue specific level, deVos *et al.* (2009) have detected an hypermethylation of the promoter region of this gene. Considering that DNA methylation is associated with gene silencing and the loss of methylation leads to overexpression of the same gene, our results reflects the same situation. Recently, Hanahan and Weinberg (2011) have highlighted that immune system plays a critical role in tumorigenesis, and indicate that leukocytes acts as tumor antagonizing but also as tumor promoters, and that this situation was largely unanticipated. Our results seem to support this idea mainly because leukocytes are the main source of genomic DNA available in a whole blood sample, and if it considered that neutrophils and lymphocytes are the most numerous cells of the circulating leukocytes, and the main cells called to infiltrate tumors (Hanahan and Weinberg 2011); and that the loss of methylation level of SEPT9 could be interpreted as SEPT9 upregulation, it is possible to hypothesize that in CRC subjects SEPT9 expression could suggest a critical immune activity related to the growing early tumor, but

without indicate if this activity is related with antagonizing or promoter behavior. On the other hand, the hypermethylated status of SEPT9 promoter at tissue specific level could be explained because cells in colon epithelium undergo division and differentiation from the cript to the villous apex, and the silencing of SEPT9 can induce cell cycle and cytokinesis alterations, considering the functions described for this gene (Peterson *et al.* 2011). However, as pointed by several Authors, SEPT9 gene corresponds to a very complex gene with several expression variants due alternative splicing (Connolly *et al.* 2011), and even when some functions have been described for it, others are expected to emerge in the future. The mechanism of action and cross-talk with different cell pathways are mainly unknown.

For NOTCH3, MSP analysis has shown statistically significant differences between studied goups. Control NOTCH3 promoter methylation level (63.32 ± 6.06) was significantly higher than adenoma (49.31 ± 6.2) ($p \leq 0.001$) and CRC (30.51 ± 12.65) ($p \leq 0.001$). Additionally, adenoma NOTCH3 promoter methylation level (49.31 ± 6.2) was significantly higher than CRC (30.51 ± 12.65) ($p \leq 0.001$). NOTCH3 promoter methylation level was extremely low (≈ 1%) in the adenocarcinoma cell model. Although the information available regarding NOTCH3 and its role in the carcinogenic process is not abundant, these results also support the concept proposed by Hanahan and Weinberg (2011) about an immune role in tumorigenesis, but suggesting an immune activity also related with adenoma formation. The low level of promoter methylation of this gene in the cell line agrees with the information reported in several publications, which indicate that NOTCH3 through NOTCH3 signaling pathway is highly expressed in normal colon-rectum epithelium (Miyamoto and Rosenberg 2011) and its overexpression is not only related with tumor growth, but also with aggressive CRC (Serafin 2011).

Finally, through multiple regression analysis was possible to establish a negative correlation between spontaneous MN frequency and methylation levels of SEPT9 and NOTCH3 promoter region (Table10). More clearly, the increase in MN frequency may correlate with the decrease in methylation level of the promoter region of these genes. In literature is not possible to find such comparison, but considering the information

previously mentioned and the available information regarding functions of these genes and how their expression lead to genomic instability, through aneuploidy phenomena, in the case of SEPT9, and to hyperproliferation in the case of NOTCH3, it could be hypothesized, as suggested by Hanahan and Weinberg (2011), that the immune system can act initially as a tumor antagonist but, because of infiltration once the damage it is well establish and the interaction with tumorigenic components of tumor microenvironment enhanced by the same immune response, it becomes to act not only as a direct tumorigenic component but also as a tumor promoter. However, the information available about this matter is poor and further studies must be conducted in order not only to answer this specific question but to investigate, for example, its role in other areas of carcinogenesis, like metastasis.

Table 7. Demographic characteristics of the study population.

	CONTROL	ADENOMA	CRC
Subjects	26	23	26
Age	59.3±6	60.43±6.14	67.69±9.76 [a, b]
Gender F/M	7/19	10/13	10/16
BMI	25.73±3.27	26.87±3.09	24.45±3.53
Smoke N/ES	19/7	18/5	18/8

Gender F/M, Females/Males; BMI, Body Mass Index; Smoke N/ES, Never/Ex or current Smoker. a) Statistically significant difference vs control group ($p \leq 0.001$). b) Statistically significant difference vs adenoma group ($p \leq 0.01$).

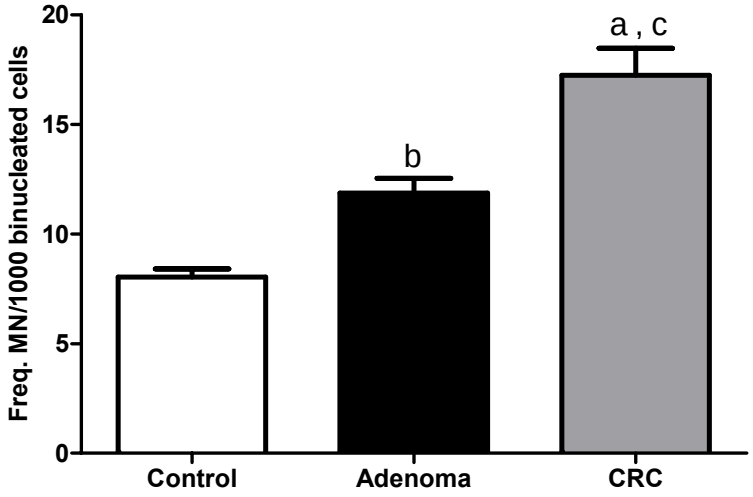

Figure 15. MN Frequency in peripheral blood lymphocytes of study groups. Mean MN ± DS; control: 8.3 ± 1.8; adenoma: 11.87 ± 3.2; CRC: 17.23 ± 6.3. Statistically significant difference vs control group ($p \leq 0.001$); b) Statistically significant difference vs control group ($p \leq 0.01$); c) Statistically significant difference vs adenoma group ($p \leq 0.001$).

Table 8. Relative Risk associated to MN frequency, age, gender, BMI and smoke habit. Control as the reference group.

GROUP	MN	AGE	GENDER	BMI	SMOKE
ADENOMA	1.71 ***	1.01	0.55	1.11	1.06
CRC	2.13 ***	1.14 *	0.94	0.95	1.58

*** statistically significant, $p \leq 0.001$. * statistically significant, $p \leq 0.05$. r^2 = 0.4.

Table 9. Influence of age, gender, BMI and smoke habit in MN formation.

Variable	β	*p*
Age	0.33	≤ 0.001
Gender	-1.9	0.12
BMI	-0.14	0.41
Smoke	2.07	0.13
Multiple Correlation Coefficient		0.538
Determination Coefficient		0.29

$p \leq 0.05$ was considered statistically significant.

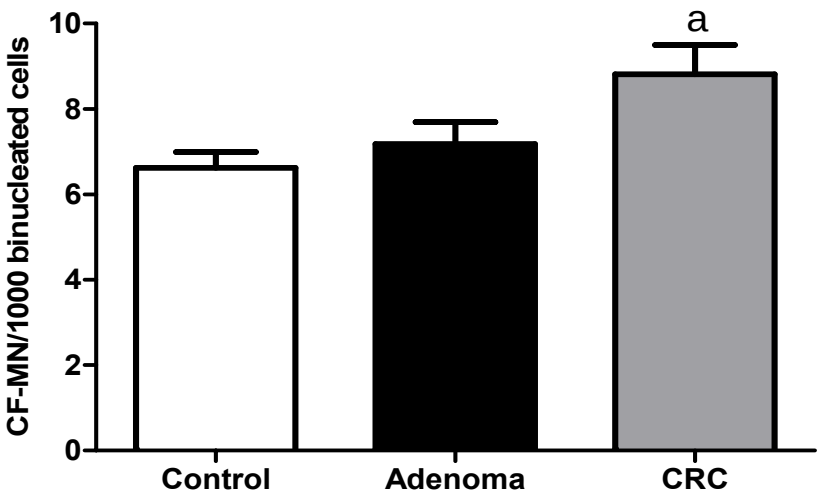

Figure 16. CF-MN Frequency induced by plasma ultrafiltrate of the studied groups over peripheral blood lymphocytes from an healthy donor. Mean MN ± DS; control: 6.6 ± 1.9; adenoma: 7.1 ± 2.5; CRC: 8.8 ± 3.5. Statistically significant difference between CRC and control group ($p \leq 0.05$).

Table 10. Influence of Age, Gender, BMI and smoke habit in MN formation induced by plasma ultrafiltrate.

Variable	β	*p*
Age	0.06	0.1
Gender	-0.59	0.4
BMI	-0.08	0.4
Smoke	-0.61	0.4
Multiple Correlation Coefficient		0.283
Determination Coefficient		0.08

$p \leq 0.05$ was considered statistically significant.

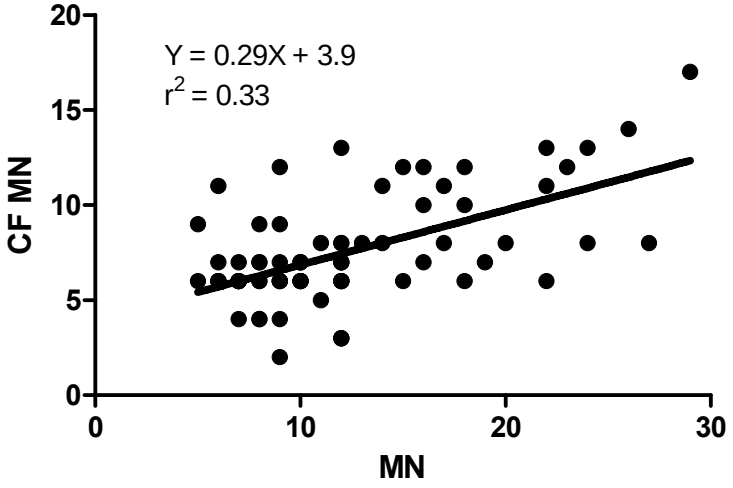

Figure 17. Linear Regression Analysis of MN Frequency and CF-MN frequency. Correlation coefficient = 0.57 ($p \leq 0.001$).

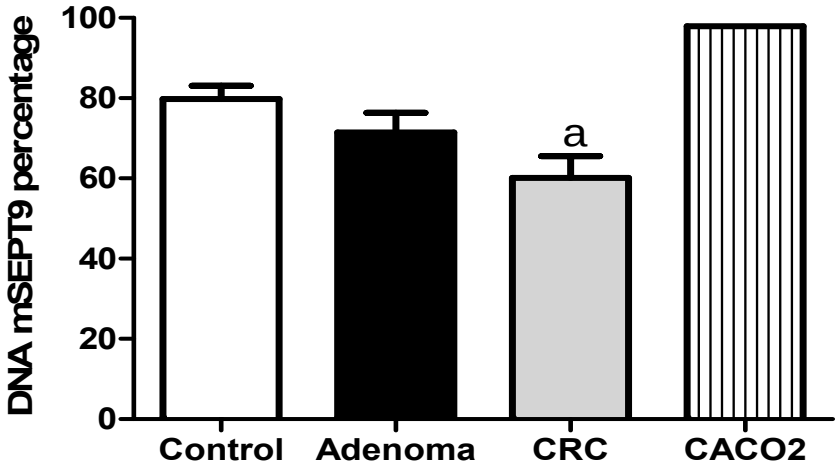

Figure 18. Whole blood DNA SEPT9 promoter methylation status among studied groups. CaCo2 cells has been included as an adenocarcinoma cell model. Control: 79.71 ± 16.24; adenoma: 71.42 ± 22.32; CRC: 60.14 ± 23.66; CaCo2: 97.94. Statistically significant difference between CRC and control group ($p \leq 0.01$).

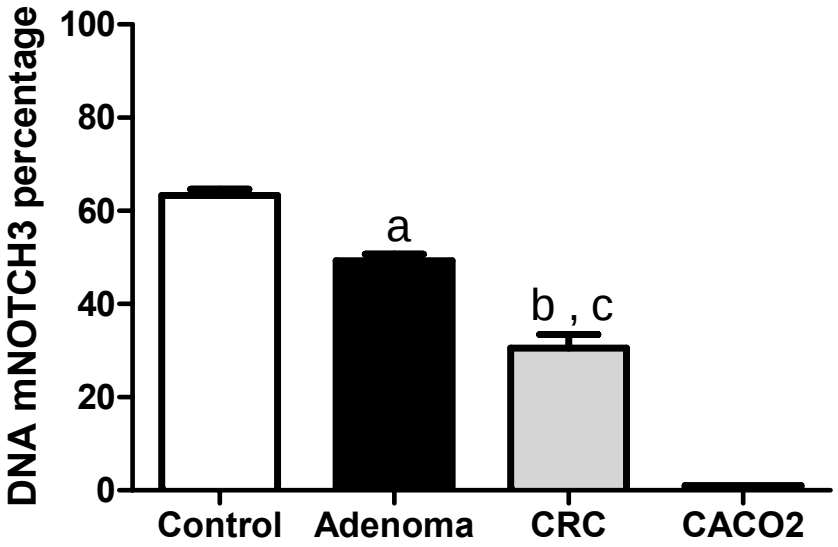

Figure 19. Whole blood DNA NOTCH3 promoter methylation status among studied groups. CaCo2 cells has been included as an adenocarcinoma cell model. Control: 63.32 ± 6.06; adenoma: 49.31 ± 6.2; CRC: 30.51 ± 12.65; CACO2: 1. Statistically significant difference between: a) adenoma and control ($p \leq 0.001$); b) CRC and control ($p \leq 0.001$); c) CRC and adenoma ($p \leq 0.001$).

Table 10. Influence of SEPT9 and NOTCH3 promoter methylation stauts on MN frequency among the studied groups.

GENE	β	*p*
SEPT9	-0.07	0.04
NOTCH3	-0.16	≤ 0.0001
Multiple Correlation Coefficient		0.61
Determination Coefficient		0.38

$p \leq 0.05$ was considered statistically significant.

5. CONCLUSIONS

CRC has been described as a leading cause for cancer-related death worldwide, but even when CRC is one in the small group of cancers which can be prevented by screening, compliance to screening test remain lower. Main efforts have been made to increase screening rates among population at risk for CRC thus the identification and development of new markers obtained from easily accessible tissues, leading to a less invasive screening test, have become of great importance.

The research has evaluated potential cellular and molecular biomarkers related with CRC risk. The results obtained allow to conclude that MN frequency varies according CRC pathologic status and, together with other variables, may be considered a valid biomarker for adenoma and CRC risk. Additionally, the plasma of patients affected with CRC not only serves as a biomarker for oxidative stress, but also as a biomarker of genetic damage correlated with the carcinogenic process that verifies in the colon-rectum.

On the other hand, it is possible to conclude that SEPT9 gene is downregulated in CRC at tissue specific level, but upregulated in whole blood cells of CRC positive subjects. In the same way, NOTCH3 is upregulated in both adenoma and CRC affected subjects, at whole blood cells level. Furthermore, SEPT9 and NOTCH3 promoter methylation status, at whole blood cells level, varies according hystopatholigal changes observed in colon-rectum, suggesting that promoter methylation status of these genes could be a reliable biomarker for CRC risk. Finally, the presence of a relationship between SEPT9 and NOTCH3 promoter methylation status and spontaneous MN frequency not only suggests a direct role of these genes in the genesis of MN, but also suggests a possible explanation for the tumor promoter activity described for the immune system.

Even if further studies must be conducted, the evidence presented supports the concept that biomarker research, at cellular and molecular level, can provide new perspectives in detection, prevention and prognosis not only for Colorectal Cancer, but for the carcinogenic process in general.

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7. ADDENDUM

Part of methods and results reported in this thesis are included in the following publications/communications:

Publcations

1. Maffei F., Angeloni C., Malaguti M., Moraga J.M., Pasqui F., Poli C., Colecchia A., Festi D., Hrelia P., Hrelia S. (2011): **Plasma antioxidant enzymes and clastogenic factors as possible biomarkers of colorectal cancer risk**. Mutat. Res. 714(1-2), 88-92.

Abstract and Comunications

1. Zolezzi Moraga J.M. "**Genetic biomarkers for the evaluation of risk assessment of colorectal cancer**". XIV Seminario Nazionale per Dottorandi in Farmacologia e Scienze affini, Siena, Certosa di Pontignano, 20-23 Settembre 2010.

2. Zolezzi Moraga J.M. "**Genetic biomarkers for the evaluation of risk assessment of colorectal cancer**", XV Seminario Nazionale per Dottorandi in Farmacologia e Scienze affini nell'ambito del 35° Congresso Nazionale della Società Italiana di Farmacologia, Bologna, 14-17 Settembre 2011.