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Common *MTHFR*, *MTR* and *MTRR* Polymorphisms in Sporadic Colo-rectal Adenocarcinoma.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professora Doutora Lina Carvalho (Universidade de Coimbra) e do Professor Doutor Paulo Santos (Universidade de Coimbra)

José Pedro Coelho Mendes

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“You can’t connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future”

Steve Jobs

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Abstract:

Folate pathway directly influences the synthesis of purins and indirectly the hypometylation of the global DNA and hypermethylation of tumour suppressor genes. Folate can modulate the risk of developing the sporadic colorectal adenocarcinoma (SCA). The intracellular folate metabolism is regulated by several enzymes including methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR) and methionine synthase reductase (MTRR). Thus, genetic changes in these genes can influence the development of SCA. Several common polymorphic SNPs (*single-nucleotide-polymorphisms*) have been reported for *MTHFR*, *MTR* and *MTRR* genes. Mutated *MTHFR*, *MTR* and *MTRR* profiles were reported in some cancer types. There are conflicting results that range between strong linkage and no association between these polymorphisms and SCA risk in different literature findings.

The aim of this study was to explore and describe the status of polymorphisms in folate-associated genes (*MTHFR C677T*, *MTHFR A1298C, MTR A2756G* and *MTRR A66G*) in SCA in relation with corresponding normal optically looking colonic mucosa.

Materials and methods: Paraffin-embedded biopsies from 100 individuals without colo-rectal (mainly reported with colitis) and 51 subjects with colorectal cancer without family history were analyzed. Genotyping of polymorphisms in the folate-associated gene *methylenetetrahydofolate reductase* (*MTHFR*, *C677T* and *A1298C*), *methionine synthase* (*MTR A2756C*) and *methionine synthase reductase* (*MTRR A66G*) were done by PCR-SSP techniques.

Results: The mutant genotypes *MTHFR* 677CT (68.6 % vs 42%; *p*=0.002 ; OR=3.021; 95% CI:1.401-6.571), *MTHFR* 1298AG (62.7% vs 26%; *p*=0.0001; OR=4.794 ;95% CI: 2.194-10.566), *MTR* 2756AG (68.6% vs 20%; *p*= 0.0001; OR=8.750; 95% CI:3.806-20.417) and *MTR* 2756GG (19.6% vs 4%; *p*=0.002; OR=5.8; 95% CI: 1.562-23.719) were found to be associated with SCA prevalence. There was also association between genotypes *MTHFR* 1298AC, *MTHFR* 1298CC, *MTR* 2756AA and *MTR* 2756 AG and colonic and rectal localization.

Conclusions: These findings provided insight into the potential pathogenesis of polymorphisms in SCA predisposal and prognostic. Dysregulation of *MTHFR*, *MTR* and *MTRR* genes is associated with decreased levels of the MTHFR, MTR and MTRR enzymes that play key roles in folate metabolism, correlated to increased SCA predisposal and colonic or rectal incidence.

Therefore, this study may contribute to increase new prevention, diagnostic and therapeutic strategies in colo-rectal carcinoma.

Key-words: Sporadic Colorectal Adenocarcinoma, MTHFR, MTR, MTRR, common polymorphisms

Resumo:

A via metabólica do folato influencia diretamente a síntese de purinas e, indiretamente, a hipometilação do DNA e a hipermetilação de genes supressores tumorais. O folato é crucial na modelação do risco de desenvolvimento de Adenocarcinoma colo-rectal esporádico (ACE). O metabolismo intracelular do folato é regulado por várias enzimas incluindo a metilenotetrahidrofolato redutase (MTHFR), metionina sintase (MTR) e a metionina sintase redutase (MTRR). Por isso, alterações genéticas nestes genes podem influenciar o desenvolvimento de ACE. Vários polimorfismos comuns têm sido descobertos nos genes *MTHFR, MTR* e *MTRR*. O perfil mutado destes genes tem sido associado a vários tipos de cancro. Há resultados contraditórios que demonstram desde uma forte associação a nenhuma associação entre estes polimorfismos e o risco de ACE em vários estudos.

O objetivo deste estudo consiste em explorar e descrever o efeito dos polimorfismos em genes associados ao metabolismo do folato (*MTHFR C677T*, *MTHFR A1298C, MTR A2756G* e *MTRR A66G*) em ACE em relação com mucosa colónica normal oticamente visível.

<u>Materiais e métodos:</u> Biópsias fixadas em parafina de 100 indivíduos sem carcinoma colo-rectal (principalmente reportados com colite) e 51 indivíduos portadores de cancro colo-rectal sem histórico familiar foram analisados. A genotipagem dos genes associados ao metabolismo do folato *metilenotetrahidrofolato redutase* (*MTHFR*, *C677T* and *A1298C*), *metionina sintase* (*MTR A2756C*) e *metionina esintase redutase* (*MTRR A66G*) foi feita pela técnica de PCR-SSP.

<u>Resultados:</u> Os genótipos mutantes *MTHFR* 677CT (68.6 % vs 42%; p=0.002 ; OR=3.021; 95% IC:1.401-6.571), *MTHFR* 1298AG (62.7% vs 26%; p=0.0001; OR=4.794 ;95% IC: 2.194-10.566), *MTR* 2756AG (68.6% vs 20%; p= 0.0001; OR=8.750; 95% IC:3.806-20.417) e *MTR* 2756GG (19.6% vs 4%; p=0.002; OR=5.8; 95% IC: 1.562-23.719) encontraram-se associados à prevalência de ACE. Também foram encontradas associações entre os genótipos *MTHFR* 1298AC, *MTHFR* 1298CC, *MTR* 2756AA e *MTR* 2756 AG e a localização colónica e rectal.

<u>Conclusões:</u> Estes resultados contribuiram para um maior entendimento na patogénese de polimorfismos implicados no risco e prognóstico de ACE. A desregulação dos genes *MTHFR*, *MTR* e *MTRR* está associada à diminuição das atividades enzimáticas de MTHFR, MTR e MTRR que têm papeis importantes no metabolismo do folato, correlacionado com o aumento do risco de ACE e com a incidência colónica e rectal. Deste modo, este estudo pode contribuir

para o aumento de novas estratégias de prevenção, diagnóstico e terapêuticas no carcinoma colo-retal.

Palavras-chave: Adenocarcinoma Colo-rectal Esporádico, MTHFR, MTR, MTRR, polimorfismos comuns.

Chapter I: Introduction

List of abbreviations:

APC: Adenomatous Polyposis Coli

CI: Confidence Interval

CRC: Colo-rectal Cancer

DCC: Deleted in Colo-Rectal Cancer

DHF: Dihydrotetrafolic acid

FFPE: Formalin-Fixed paraffin-Embedded

Hcy: Homocysteine

HNPCC: Hereditary Nonpolyposis Colon Cancer

MTHF: Methytetrahydrofolate

MTHFR: Methyltetrahydrofolate Reductase

MTR: Methionine Synthase

MTRR: Methionine Synthase Reductase

OD: Optical Density

OR: Odd Ratio

PCR-SSP: Polymerase Chain Reaction-Single Specific Primers

RR: Relative Risk

SCA: Sporadic Colo-rectal Adenocarcinoma

SNP: Single Nucleotide Polymorphism

TAE: Tris-Acetate-EDTA

THF: Tetrahydrofolate

UV: Ultra-Violet

WHO: World Health Organization

Introduction

Cancer is characterized by uncontrolled growth and spread of mutated cells. External factors (for example, alcohol and radiation) and internal factors (such as hormones and mutations) may trigger initiation and promotion of carcinogenesis. When progression is uncontrolled, it can result in fatality. However, prevention can be achieved in certain types of cancer by inhibiting the effects of these factors.

Diseases are most treatable when there is an early diagnosis or before cells become malignant. It can be treated with different strategies such as chemotherapy, immunotherapy, surgery, radiation and hormones. Research shows that environmental factors, such as diet, influence gene expression control mechanisms (for example, epigenetic processes) that can eventually lead to the development of malignant disease.

Studying the impact of nutrients on genes and their encoded proteins and the influence of genetic factors on diet is essential for the development of strategies of prevention. Certain food components change the predisposition to cancer (Manson et al., 2007; Adams et al., 2008), therefore, it is needed a greater understanding of interactions between diet and disease in order to determine new therapeutics of prevention.

Nutrition has been essential in prevention and treatment of cancer (Jones et al., 1997; Lamprecht et al., 1996; Lifshitz et al., 1998). The human genome, composed by 3 billion bases, is shared by 99,9% of the individuals and a small existing difference is explained by variations of single nucleotide polymorphisms (SNPs) (Potten et al., 1992; Johnson et al., 1992) and by copy number variation which consists on gains and losses of segments of DNA, therefore, influencing variation in biological responses and risk of disease. Conventional medicine attempts to use the information within these variations and compare them with the response to different diets and predisposition to the disease (Jones et al., 1997; Lamprecht et al., 1996; Lifshitz et al., 1998; Potten et al., 1992; Potten et al., 1997; Pritchard et al., 1996; Reed et al., 1998; Thompson et al., 1995; Thompson et al., 1992), based on the fact that the genetic constitution of each

individual changes the response to different bioactive food components, contributing to the determination of the relative risk of several pathologies, such as Colorectal Cancer (CRC) (Smith et al., 2001).

1- Colorectal carcinoma (CRC):

1.1- Incidence and mortality:

Colorectal cancer (CRC) is one of the most prevalent tumours of nowadays, particularly in developed countries. Worldwide, this tumour represents the third major cause of death in men and the second in women (Garcia et al., 2007; Center et al., 2009; Globocan 2012).

Based on statistics from data of 2012 (Globocan 2012), CRC is responsible for 9% of death by cancer in United States of America (USA) and for 13% in Europe (Globocan 2012). In Portugal, statistics show that CRC is the most frequent type of tumour in similar localizations for both genders (1 out of 6 diagnosed malignant tumours), being the second cancer with the most significant incidence to men and women. The rate of mortality associated to this tumour is high, contributing to approximately 16% of the total oncologic mortality rate in Portugal (Globocan 2012). Mortality by CRC has increased approximately 80% during the last two decades of the 20th century (Mendes et al., 2008). Approximately 10%-30% of all CRC cases occur in the context of a family history (such as Lynch Syndrome) (Smith et al., 2001), but the predisposing genetic factors are still unknown. Familial adenomatous polyposis (FAP), MYH-associated polyposis (MAP), and hereditary nonpolyposis colon cancer (HNPCC), which are highly penetrant and inherited CRC syndromes, are less common examples, accounting for up to 5% of CRC cases (Kwak et al., 2007). In spite of the familial cases, the majority of CRC cases occur occasionally, indicating that health behaviors are strongly correlated to disease development (Qi Dai et al., 2007; Sehitoglu et al., 2014).

1.2- Worldwide Distribution:

CRC is more common in developed countries whereas in African and Asian continents is low, except in Japan, where the incidence has increased and approaches that of the West (figure 1). The interaction between dietary and other environmental factors with genetic factors can explain the difference in incidence between geographic regions.

Studies from migrant populations from areas of low incidence to high incidence areas, report acquisition of the disease risk of the host country over time, such as the case of Hispanic and Japanese populations migrating to the United States. In addition, there are evidences that ethnics influence incidence of CRC; for example, mutations in damage repair genes of DNA are more frequent in Afro-Americans (Weber et al., 1993).

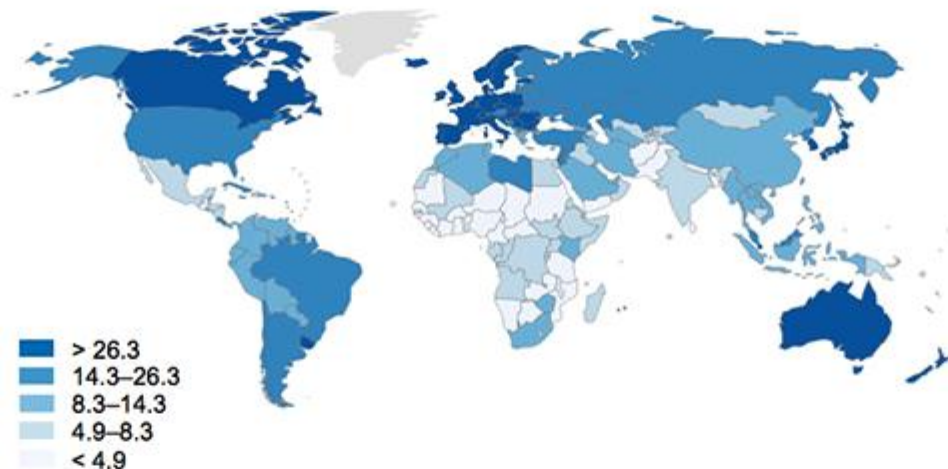

Figure 1: Worldwide colorectal cancer incidence rates for both genders. (**Adapted from:** http://www.crcprevention.eu/index.php?pg=colorectal-cancer-epidemiology)

1.3- Epidemiology and CRC:

There are three known types of this disease: sporadic forms, inherited syndromes and family forms (Willet et al., 1989; Calvert et al., 2002). Approximately 70% of CRCs correspond to sporadic forms of disease that follow the suppressing pathway of carcinogenesis, according to Fearon et al. (Fearon et al., 1990). This pathway is characterized by accumulation of mutations and allele losses in several tumour suppressor genes (Fearon et al., 1990). The remaining 30% have a strong inherited influence (147); 5% of these result in mutations highly associated to specific genes. The presence of these mutations leads to the development of two distinct syndromes: familial adenomatous polyposis and non-associated polyposis colon inherited carcinoma (Fantuzzi et al., 2005). The first one is characterized by an excess of adenomas all the way through colon; the cause of this syndrome resides in a germinal

mutation in *APC* gene (Giardello et al., 1997). On the other hand, the non-associated polyposis colon inherited carcinoma, known as Syndrome of Lynch, does not show a usual phenotypic expression and its developments results from germinal mutations in genes involved in DNA repair (Leach et al., 1993). The accumulation of these mutations results from an inactivated repair system, and this is defined as the mutant pathway of carcinogenesis (Fishel et al., 1993).The remaining 25% correspond to family forms of CRC whose mechanisms are not yet well known, either by a clinical perspective, either by a molecular point of view (Castells et al., 2009; Ferreira et al., 2009). Therefore, the risk of developing CRC in individuals without a family history of CRC and whose ages are higher than 50years old is 5-6%. When there is a medical history of CRC in a relative of first and second degree the risk increases to 20%, and in individuals who present one of the syndromes referred before, the risk reaches 80-100% (Rutgi et al., 2007).

Other important CRC type is Sporadic Colorectal Carcinoma (SCA). Its development is intimately associated to at least two classes of genes: Oncogenes, involved in regulation of cell proliferation, and tumour suppressor genes, negative regulators of cell proliferation. Gain-of-function mutations (Alberts et al., 2006) in oncogenes are dominant, whereas loss-of-function mutations (Grady e Markowitz, 2002) in tumour suppressor genes are recessive, and both lead to altered cell proliferation. In SCA, random mutations during carcinogenesis follow a well-established sequence. The inactivation of *APC* gene located in chromosome 5 (5q) is the first step to dysplasia in an adenoma. Additional mutations accumulate in oncogenes (*RAS* family) and tumour suppressor genes in chromosomes 18q (*DCC*, *SMAD2*, *SMAD4*) and 17q (*TP53*), leading to an accentuated dysplasia and subsequently to carcinoma (Fujiwara et al., 1998).

1.3.1- <u>Histological classification:</u>

SCA is the most common histological form of CRC (90%). In 1925, Albert Broders was the first to classify adenocarcinomas by its staging system of differentiation, based on the percentage of differentiated cells (Broders, 1925). Later in 1932, Cuthbert Dukes changed Broders classification and included three subdivisions in a new system of differentiation: stage I (well differentiated), stage II (moderately differentiated) and

stage III (undifferentiated) (Dukes, 1932). The histological staging is universally accepted as a prognostic factor in CRC (Compton et al., 2000). The histological classification of CRC in this study was based upon the most recent classification version (7th edition, 2012) according to World Health Organization (WHO) (table 1).

Histological classification according to WHO			
Epithelial tumours		**Non-epithelial tumours**	
Adenoma		Lipoma	
	Tubular	Leiomyoma	
	Villous	Gastrointestinal stromal tumour	
	Tubulovillous	Leiomyosarcoma	
	Serrated	Angiosarcoma	
		Kaposi sarcoma	
Intraepithelial neoplasia2 (dysplasia)		Malignant melanoma	
associated with chronic inflammatory diseases		Others	
	Low-grade glandular intraepithelial neoplasia		
	High-grade glandular intraepithelial neoplasia		
		Malignant lymphomas	
Carcinoma			Marginal zone B-cell lymphoma of MALT Type
	Adenocarcinoma		Mantle cell lymphoma
	Mucinous adenocarcinoma		Diffuse large B-cell lymphoma
	Signet-ring cell carcinoma		Burkitt lymphoma
	Small cell carcinoma		Burkitt-like /atypical Burkitt-lymphoma
	Squamous cell carcinoma		Others
	Adenosquamous carcinoma		
	Medullary carcinoma		
	Undifferentiated carcinoma	**Secondary tumours**	
		Polyps	
Carcinoid (well differentiated endocrine neoplasm)			
	EC-cell, serotonin-producing neoplasm	Hyperplastic (metaplastic)	
	L-cell, glucagon-like peptide and PP/PYY producing tumour	Peutz-Jeghers	
	Others	Juvenile	
	Mixed carcinoid-adenocarcinoma		
	Others		

Table 1: Histological classification according to the 7th version (2012) from World Health Organization (WHO).

2- CRC Risk Factors:

The origin of CRC is multifactorial due to its own ethiopathogenesis, the complex interaction between environment factors and genetic susceptibility. The initiation stage evolves from one or more genetically altered cells and gradually develops allowing nutrients to modify the evolution of the disease. Nutrients contact epithelial cells in the colon tissues, thereby presenting impact on development mechanisms in both normal and transformed epithelial cells of colon. (Potten et al., 1997) Recent research has shown a solid link between inadequate folate (one of the essential B vitamins) intake and increased risk of CRC, namely when associated with genetic factors.

2.1- Environment Factors:

The types of diet and environment features have been recognized as modulating factors of the risk of development of this type of cancer (Black et al., 1997; Visser et al., 2012; Hall et al., 1994; Jones et al., 1997). Several studies suggest the existence of distinct bioactive food components responsible for the more or less incidence (Visser et al., 2012; Hall et al., 1994; Jones et al., 1997). These components can be controlled and/or adjusted to each individual (Lamprecht et al., 1996; Lifshitz et al., 1998). Although consistent associations are present between CRC and certain diet patterns, conflicting results have also been found (Potten et al., 1992; Potten et al., 1997). These discrepancies may be reflected on: the multifactorial and complex nature of the disease; the capacity of regulation of several genetic pathways of each nutrient; and the genetic heterogeneity of the populations under study (Potten et al., 1992; Potten et al., 1997; Potten et al., 1997). Also designated as modifiable factors, environment factors implied in CRC are related to body composition, physical exercise and dietary patterns.

2.1.1- Dietary Pattern:

It is known that nutrition plays an important role on prevention of this type of tumour; in developed countries, more than 50% of CRC cases are attributed to dietary causes (Huxley et al., 2009; Bingham et al., 2000; Howe et al., 1992). According to the World Cancer Research Fund/American Institute for Cancer Research, several associations have been found, with higher or lower evidence, between food components/nutrients and the risk of CRC (World Cancer Research Fund/American Institute for Cancer Research, 2007). When it comes about foods, high consumption of red meat, processed meat and alcoholic beverages are significantly associated to a higher risk of CRC (World Cancer Research Fund/American Institute for Cancer Research, 2007). Foods such as garlic and milk appear to present a protective trend in the risk of this type of carcinoma (World Cancer Research Fund/American Institute for Cancer Research, 2007). In the case of nutrients, fiber and calcium show a more evident role in protection against CRC. Folate, vitamin D and selenium also show a strong link with protective effect. On the other hand, the opposite is observed in iron, sugars and animal fat, without revealing associated evidence (World Cancer Research Fund/American Institute for Cancer Research, 2007).

According to the World Cancer Research/American Institute for Cancer Research, foods rich in folate do not have a clear protective role against CRC (World Cancer Research Fund/American Institute for Cancer Research, 2007). The protection of this vitamin is referred for the first time by Freudenheim et al. (Freudenheim et al., 1991), observing that a diet with low levels of folate and methionine and high levels of alcohol are associated to an increased risk of CRC (Sanjoaquin et al., 2005; Flood et al., 2002;Konnings et al., 2002; Glynn et al., 1995). Folate is obtained in diet through consumption of vegetables, oleaginous fruits or cereals. The mechanism of this vitamin may influence the process of carcinogenesis which appears to be related with its participation either on synthesis and methylation of DNA (Choi et al., 2002; Lucock et al., 2005). Folate circulates on bloodstream by the form of 5-methyltetrahydrofolate (5-MTHF), which is the source of methyl groups necessary to the synthesis of nucleotides essential to DNA repair and synthesis. Also, it acts as a methyl group donor

during remethylation of homocystein into methionine, which subsequently is metabolized in S-adenosylmethionine (SAM), the universal methyl group donor, which is fundamental to methylation of cytosine in DNA. The deficiency of folate promotes and accelerates the process of carcinogenesis by changing these processes. A reduced ingestion of this vitamin, consequently changes the synthesis of DNA precursors and alters DNA repair mechanisms. When a diet is poor in folate, uracil is incorporated in the DNA, instead of thymine, resulting in DNA strand breakage (Duthie et al., 2010). Altered levels of methylation of cytosine lead to DNA methylation, promoting the overexpression of several genes, including oncogenes, which lead to cell proliferation dysregulation (Duthie et al., 2010; Ryan et al., 2001). Hypomethylation also leads to breaks in the DNA strand, originating mutations that contribute to altered gene expression involved in CRC carcinogenesis (Fearon et al., 1990). Kim et al. showed that the protection associated to folate consumption was only observed when consumptions were higher than 409µg/day, which is similar to the value (400µL/day) established by National Academy of Sciences (Food and Nutrition Board, 2002).

The Mediterranean diet is composed by foods, such as cabbages, meat and yeasts, that are rich in folate and, therefore, it has been suggested as benefic choice to decrease cancer incidence (Giacosa et al., 2013). However, there are not still enough studies that report the real role of these foods in prevention of the disease.

2.2- <u>Genetic Factors:</u>

Genetic factors clearly play a role in CRC development and predisposition, reflected by an increased risk of cancer between the family members of patients or offspring. In the presence of certain environment factors, such as, alcohol, only a few individuals will develop CRC, possibly due their genetic profile. Polymorphisms may induce a modification of a key amino acid in the encoded protein sequence, altering protein function/activity and, thus, the pathway or process involved. Alterations in the gene promoter sequence may change the promoter activity, influencing gene transcription

and resulting in altered encoded protein level and consequently changed function (Ross et al., 2010).

The strength of the biological impact will also depend on heterozygosity or homozygosity of the variant allele (Wettergren et al., 2010). Several polymorphisms are described as susceptible genes or as essential genes to DNA methylation, such as Methylenetetrahydrofolate Reductase (*MTHFR* C667T and A1298C), Methionine Synthase (*MTR* A2756G) and Methionine Synthase Reductase (*MTRR* A66G), which by interaction with certain nutrients, seem to modulate the risk of CRC.

2.2.1- <u>Folate metabolism genes:</u>

Folic acid (pteroylglutamic acid-vitamin B9) is the most stable form of folate and it is one of the essential substrates for the cell metabolism (synthesis of DNA and methylation). However, it is not naturally found in living tissues, therefore it requires *in vivo* reduction, resulting in dihydrofolate and tetrahydrofolate, by addition of atoms of hydrogen (figure 2). Its deficiency has been associated with several malignancies, including CRC.

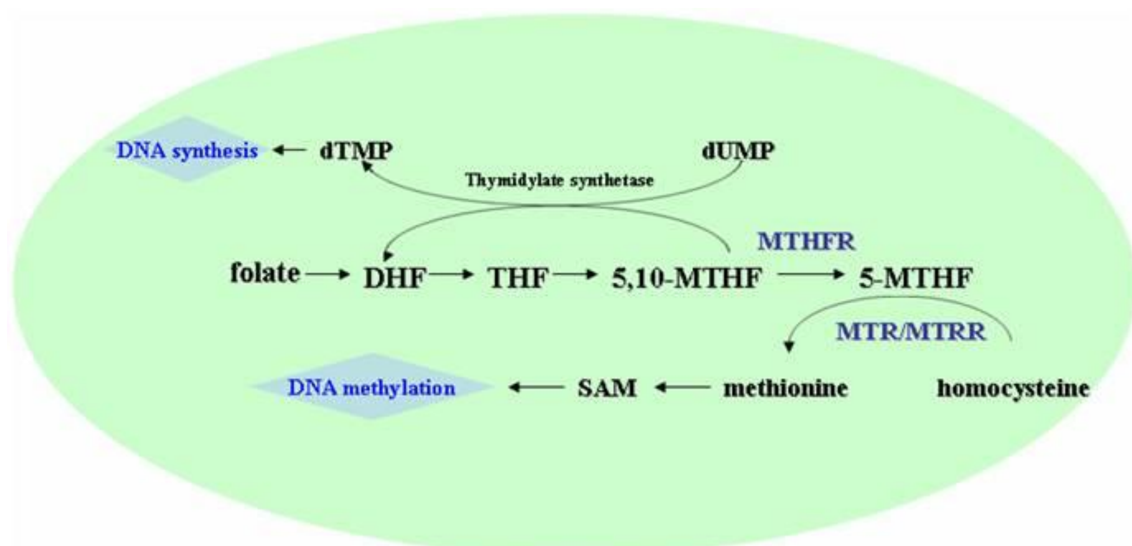

Figure 2: The metabolism of folate. DHF, dihydrotetrafolic acid; THF, tetrahydrofolic acid; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolat; SAM, S-adenosylmethionine; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine

synthase; MTRR, methione synthase reductase (**Adapted from:** http://www.ijbs.com/v08p0819.htm)

Methylated derivates of folic acid (5,10-MTHF) are carbon donors to the synthesis of nucleotides. 5-MTHF, which is another derivate of folic acid, generated from 5,10-MTHF by the MTHFR enzyme, is a methyl donor from methionine (Met) during the synthesis of homocysteine (Hcy) (figure 2). This reaction is catalyzed by methionine synthase (MTR), whose cofactor is cobalamine (vitamin B12) (Stryer, 1992). The activity of methionine synthase depends on the function of methionine synthase reductase (MTRR), which participates in the exchanging process of MTR to its active form (Leclerc et al., 1998) (figure 2). Therefore, the folate status can potentially be affected by these polymorphic genes.

2.2.1.1- Methylenetetrahydrofolate Reductase (MTHFR)

In 1994, the encoding gene of the human enzyme MTHFR was identified. It is located in the chromosome 1 (1p36.3) and it has 11 exons, coding 656 residues of aminoacids (Goyette et al., 1998) (figure 3). The substrate of MTHFR is 5,10-MTHF and generates 5-MTHF (the active form of folate in plasma) by its action (Choi et al., 2002). The 5-MTHF is the essential methyl group donor that allows the methylation of homocystein into methionine. Methionine is the precursor of S-adenosylmethionine (SAM), the provider of methyl groups to the majority of the methylation reactions of DNA (Giovannucci et al., 2003; Cohen et al., 2003; Choi et al., 2002; Lucock et al., 2005). In addition, the substrate of the enzyme, 5,10-MTHF, is an important cofactor to the *de novo* synthesis of its own nucleotides. Thus, genetic changes in the *MTHFR* gene can influence the risk of several pathologies (Shrubsole et al., 2006; Duthie et al., 2011), such as CRC. Two common polymorphisms have been reported in exons 4 (codon 677 *C<T*) and 7(codon 1298 *A<C*) of *MTHFR* gene.

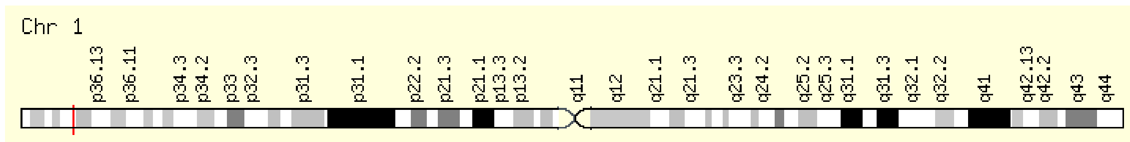

Figure 3: Genomic view for *MTHFR* gene. (**Adapted from:** http://v4.genecards.org/cgi-bin/carddisp.pl?gene=MTHFR&UserNotification=BetaRedirect)

The *MTHFR* C677T polymorphism (rs1801133) leads to an alanine-to-valine conversion (Goyette et al., 1998). Brattstrom et at. showed that approximately 10% and 50% of Caucasians presented the variant homozygous genotype *MTHFR* 677TT and variant heterozygous 677CT, respectively (Brattstrom et al., 1998). Individuals with homozygous *MTHFR* 677TT genotype have 30% enzyme activity and heterozygous carriers show 65% (Frosst et al., 1995). This genotype is also associated with higher plasma homocysteine and reduced plasma folate levels, (Frosst et al., 1995) leading to genomic DNA hypomethylation (Ulrich et al., 2005) concomitant with gene-specific promoter hypermetylation (Oyama et al., 2004), as it is frequently observed in cancer. There seems to be statistically significant associations between CRC risk and *MTHFR* C77T polymorphisms since it expresses a rate-limiting in the methyl cycle.

The polymorphism *MTHFR* A1298C (rs1801131) located in the encoding region of the regular domain, leads to a glutamate-to-alanine substitution at codon 429, (van der Put et al., 1998; Chen et al., 2002; Weisberg et al., 2002). The variant homozygous *MTHFR* 1298CC and heterozygous *MTHFR* 1298AC were identified in approximately 10% and 15% of the individuals, while the frequency of the variant allele C is approximately 36% (Botto and Yang, 2000). This polymorphism may result in a reduction of 40% in enzyme activity. Nevertheless, this variant has not been associated either with a thermolabile enzyme or with alterations in the levels of homocysteine in the plasma (Weisberg et al., 2002). Two metanalyses suggest that the variant homozygous genotype *MTHFR* 1298CC protects individuals from CRC (Kono and Chen, 2005; Huang et al., 2007), in spite of the functional effect of the variant allele C still being unknown. There have been reported statistically significant associations between CRC risk and *MTHFR* A1298C polymorphisms in several studies.

Both mutated *MTHFR* gene profiles were reported in some cancer types (Izmirli et al., 2011; Izmirli et al., 2013). There are conflicting results that range from strong linkage

to lack of association between *MTHFR* and CRC risk in different literature findings. Conflicting results in different studies can be explained to differences of diet habits, racial variations and lifestyle, besides the fact that eventually other polymorphisms can be involved in the process of carcinogenesis.

2.2.1.2- <u>Methionine Synthase (MTR):</u>

The human gene that codes the enzyme MTR was located in the telomeric region of chromosome 1 (1q43) (figure 4). The gene is constituted by 12 exons and codes a protein of 1265 aminoacids (Chen et al., 1997; Leclerc et al., 1998; Mathews et al., 1998). Methionine synthase gene (*MTR*) encodes other key enzyme involved in the folate-mediated one- carbon metabolism. It catalyzes the methylation of homocysteine to methionine with simultaneous conversion of 5-MTHF to tetrahydrofolate (THF). MTR is essential for the provision of SAM, as well as the provision of THF for use in synthesis of nucleotides (Banerjee et al., 1990).

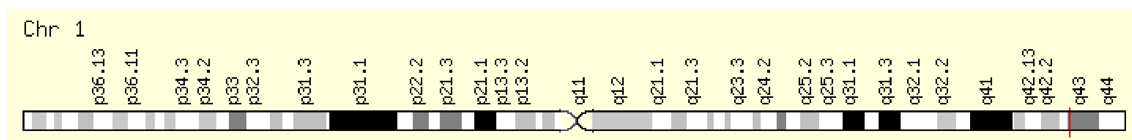

Figure 4: Genomic view for *MTR* gene. (**Adapted from:** http://v4.genecards.org/cgi-bin/carddisp.pl?gene=MTR&UserNotification=BetaRedirect)

MTR is also essential for maintaining intracellular folate homeostasis, avoiding toxic levels of homocysteine (Van Der Put et al., 1997). Significant low levels of MTR may also result in an accumulation of 5-MTHF and deficiency of intracellular folate derivatives including 5,10-MTHF required for thymidylate biosynthesis leading to deoxynucleotide pool imbalances (Manson et al., 2007; Ayala et al., 2009; Black et al., 1997). This leads to the accumulation of deoxyuridylate in DNA, possibly leading to strand breaks commonly seen in CRCs (Visser et al., 2012).

A common *MTR* variant is an A-to-G transition at base-pair 2756 (rs 1805087) in the protein-binding region of MTR, resultant from a point mutation which involves the aspartic acid-to-glycine substitution at codon 919, located in exon 8 (Van Der Put et al.,

1997; Van Der Put et al., 1998). The variant homozygous genotype *MTRR* 2756GG was identified in 2% to 6% of the individuals (Sharp e Little, 2004). *MTR* A2756G gene polymorphism was initially thought to be associated with lower enzyme activity, leading to homocysteine elevation and DNA hypomethylation (Lamprecht et al., 1996; Lifshitz et al., 1998); however, some recent studies reported a higher enzyme activity in the mutated forms (Lifshitz et al., 1998). Tumours occurring in the *MTR* 2756GG genotype show a low number of hypermethylated *CpG* islands of tumour-suppressor genes. This genotype has also been associated with an increased risk of CRC (De Vogel et al., 2009).

2.2.1.3- Methionine synthase reductase (MTRR):

MTRR (5-methionine synthase reductase) is also an important enzyme required for protein synthesis and folate metabolism. This enzyme is coded by the gene *MTRR,* which is located in chromosome 5 (5p15.31) (Leclerc et al., 1999) (figure 5). MTRR is responsible for the regulation of MTR. After a period of being turned on (active), MTR turns off (becomes inactive) and MTRR is needed to reactivate MTR in order to continue to produce methionine.

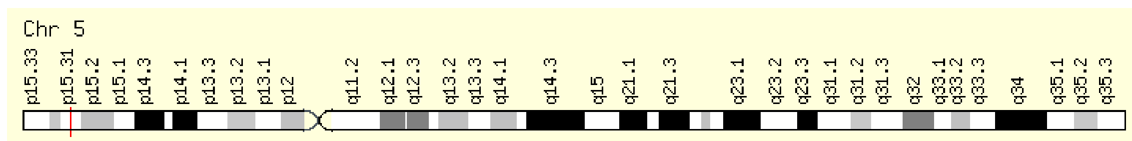

Figure 5: Genomic view for *MTRR* gene. (**Adapted from:** http://v4.genecards.org/cgibin/carddisp.pl?gene=MTRR&UserNotification=BetaRedirect)

The most frequent polymorphism in *MTRR* gene is *MTRR* A66G (rs1801394) that involves the isoleucine-to-methionine substitution (I22M), at codon 22. The variant homozygous *MTRR* 66GG was identified in 19% to 29% of global individuals. (Wilson et al., 1999; Hobbs et al., 2000; Gaughan et al., 2001, Geisel et al,. 2001; Ray et al., 2001; Hassold et al 2001, O'Leary et al., 2002, Le Marchand et al., 2002; Rady et al., 2002). It

has been demonstrated that the genotype *MTRR* 66GG is inversely associated with higher plasma homocysteine levels (Leclerc et al., 1998; Gaughan et al, 2001), resulting in a variant protein exhibiting a 4-fold lower activity compared with the wild-type protein *in vivo* (Olteanu et al., 2002). Thus, the *MTRR* 66G variant allele decreases the availability of SAM by reducing the level of active MTR. Consequently, DNA hypomethilation may be induced. It has been reported that patients heterozygous for the *MTRR* A66G polymorphism showed a reduced risk of CRC recurrence (Le Marchand et al., 2002).

3- Folate Metabolism Genes and CRC:

Metabolism of folate plays an important role in maintaining the integrity of DNA through the synthesis of nucleotides and by mechanisms of methylation (Choi et al., 2000) (figure 6). The folate pathway directly influences the synthesis of purines and indirectly the hypometylation of the global DNA and hypermethylation of tumour suppressor genes, such as *APC* and *p16* (Poirier et al., 1994; Manson et al., 2007; Lulock et al., 2005). Therefore, the main enzymes of this metabolism are MTHFR, MTR and MTRR. All the enzymes involved in the metabolism of folic acid, methionine and homocysteine are coded by polymorphic genes, which are responsible for changes in the activity of key enzymes (Frosst et al., 1995; Goyette et al., 1998; Van Der Put et al., 1997; Chen et al., 1997; Leclerc et al., 1998; Ulrich et al., 2002). In conclusion, folate and folate metabolism genes seem to be of special interest to colon and rectal cancer, as they are involved in DNA maintenance, repair and methylation, and recent research suggests that *MTHFR, MTR* and *MTRR* are key genes in CRC.

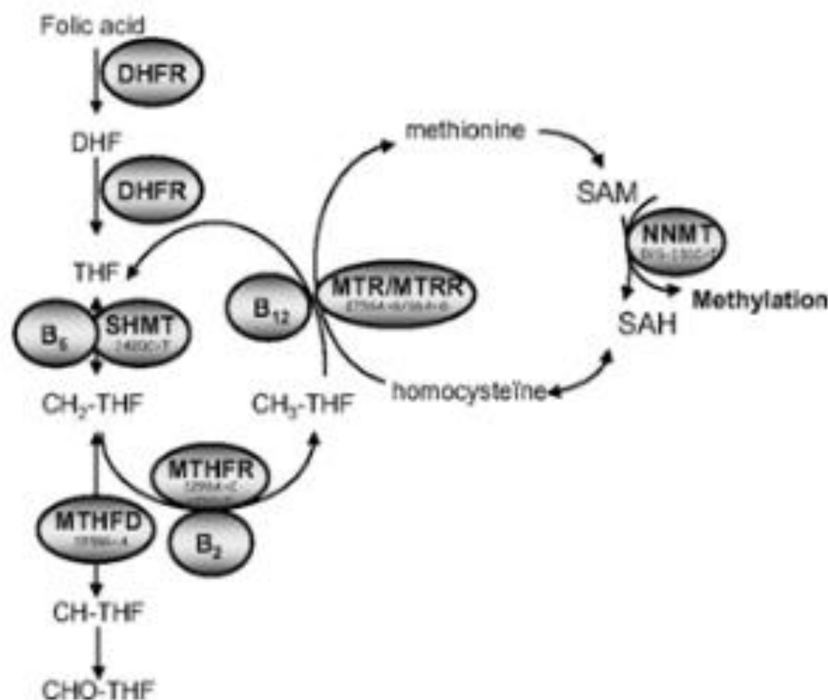

Figure 6: Simplified scheme depicting intracellular metabolism of folates and its role on methylation process. (**Adapted from:** Jonge et al., 2009)

4- Objectives:

A group of biopsies of Sporadic Colorectal Adenocarcinoma (SCA) and a group of biopsies collected in individuals with nonspecific colitis and normal mucosa included in the biopsies, (control group) were assessed in order to:

-Verify if *MTHFR* C677T, *MTHFR* A1298C and *MTRR* A66G polymorphisms influence the risk of occurrence of SCA;

-Verify if the studied polymorphisms are associated to gender and colonic and rectal localization among SCA subjects.

This study was developed according with the Faculty of Medicine ethical rules for Retrospective Studies.

Chapter II: Materials and methods

5- Materials and methods

5.1 – Biological Samples:

A global of 51 samples of SCA (mean age of 73.7 +/-10.7 years; 80.4% men and 19.6% women) was used in the research of the defined polymorphisms (table 2). A total of 28 colon and 14 rectal biopsies formalin-fixed paraffin-embedded (FFPE) were selected. A total of 100 biopsies of otherwise healthy subjects were used as control group (mean age of 72.7 +/-8.9 years; 79% men and 21% women) (table 2).

The biopsies were randomly collected from 2009 to 2011 from the archives of Institute of Anatomical Pathology, Faculty of Medicine of the University of Coimbra. SCA samples were clinically identified as belonging to colon and rectum carcinomas and were selected according to the malignant cells availability with at least 60 malignant cells. The SCA cases were classified according to WHO criteria (table 2). This study was supported and approved by local ethics committee (CIMAGO - Faculty of Medicine of the University of Coimbra, Coimbra, Portugal).

		Distribution	Age		Tumour localization				
					Colon		Rectum		**Total**
		%	Mean	Stand. Dev.	n	%	n	%	
	Gender								
Patients	Male	80.4	73.7	10.7	27	81.8	14	77.8	41
	Female	19.6			6	18.2	4	22.2	10
	Total	100			33	100	18	100	51
Controls	Male	79	72.7	8.9					
	Female	21							
	Total	100							

Table 2: Clinical pathological data SCA and controls.

5.2 - DNA extraction:

According to the protocol of extraction from *NZY Tissue gDNA Isolation Kit* (NZYTech, Lisbon, Portugal) of paraffin embedded tissues, after microdissection of the paraffin block, 5 to 10 dissections of 10µm of thickness from each sample were obtained, posteriorly adding xylene to the removal of paraffin. After centrifugation, the samples were washed in ethanol (96%-100%). Lysis of the bacterial wall by Proteinase K was then performed overnight and purification of DNA obtained through columns and according to the procedure of the referred kit. In the end of the step, we proceeded to the determination of the purity and concentration of DNA of all the samples, by spectrophotometry, applying a wavelength of 260/280nm.

5.2.1 - Analysis of concentration and quality of the extracted DNA:

DNA samples were quantified in a spectrophotometer *GeneQuant pro* (Biochrom, Cambridge, England). Rnase-free water was applied as reference and 7 µl of DNA sample were inserted in the ultra-microvolume *cuvette* in order to perform the quantification and measurement of concentration and purity of the sample, by reading adequate optical densities (230 nm, 260 nm and 280 nm). The existence of nucleotides and proteins was detected at a wavelength of 280nm, while at the 260 nm wavelength there is detection of nucleotides only. At a wavelength of 230 nm, the presence of contaminants is assessed.

5.3 - Genotyping:

The genotyping of polymorphisms was carried through commercial kits "Nutri Box Kit" (Genebox, Cantanhede - Portugal) using PCR-SSP technique. These kits included internal, negative and positive controls for each sample. The detection of *MTHFR C667T, MTHFR A1298C,*

and *MTR A2756G* and *MTRR A66G* mutations was performed using manufacturer protocol (table 3). The amplified PCR products was analysed by electrophoresis with a SYBR Safe (Molecular Probes, Oregon – USA) in 2% agarose gel and visualized in a ultra-violet (UV) transilluminator (UVi Tech, Cambridge, United Kingdom).

Step	Temperature	Time	Number of cycles
Initial denature	95º C	1 Min	1
Denature Annealing Extension	95º C 70º C 72º C	25 Sec 45 Sec 30 Sec	5
Denature Annealing Extension	95º C 65º C 72º C	25 Sec 45 Sec 30 Sec	21
Denature Annealing Extension	95º C 55º C 72º C	25 Sec 1 Min 2 Min	4
Final extension	72º C	10 Min	1

Table 3 – Protocol of amplification by PCR-SSP (Polymerase Chain Reaction – Single Specific Primers), from Genebox, Cantanhede – Portugal.

5.3.1 - Electrophoresis in agarose gel:

After amplification, PCR reactions were submitted to electrophoresis in 2% agarose gel in order to identify the amplified products. Agarose *Routine Grade* (NZYTech, Lisbon, Portugal) was dissolved in TAE (Tris-acetate-EDTA) (NZYTech, Lisbon, Portugal) and distillated water and agitated for 15 seconds. Then the solution was transferred to the microwaves device to complete dissolution of agarose. Afterwards, we took the Erlenmeyer out of the microwaves in order to cool it down, followed by the addition of $1x10^{-5}$ SYBR Safe (Molecular Probes, Oregon – USA), a dye that allows the visualization of DNA under the UV light incidence, and then agitated for 15 seconds for homogenization.

The solution was collocated onto the mold until it solidifies under the environment temperature. After solidification, the solidified gel was inserted in the plastic gel box, previously filled with 1x TAE (NZYTech, Lisbon, Portugal). The PCR samples were then inserted into the gel wells and they were left running for 10 minutes under the 300 volts of the *Power Pac Basic* device (Bio-Rad, California, USA). Finally, the PCR products were visualized under UV light by a transilluminator (UVi Tech, Cambridge, United Kingdom). The results were further registered by a digital camera (NIKON DMX1200F).

5.4 - Statistical analysis:

5.4.1 - Analysis of purity and concentration of DNAs:

The study of purity and concentration of DNAs consisted on the calculation of the means, standard-deviations and confidence levels relatively to their concentrations and quantity of proteins. This statistical analysis aimed to verify if the conditions of the DNAs were acceptable for the validation of the results in the study.

5.4.2 - Allele and genotype frequencies of polymorphisms:

Allele and genotype frequencies were calculated from obtained percentages for each studied polymorphism. In order to assess if the mutations were in equilibrium, *chi*-square test was performed using the Hardy-Weinberg equilibrium as reference. *MTHFR C667T, MTHFR A1298C, MTR A2756G, MTRR A66G* frequencies were compared between different groups (SCA versus control and tumour localization) using STATISTICA 14 (StatSoft, Inc., 2013) based on *chi*-square (2x2) test and Exact Fisher test. The significance level was set at $p<0.05$, odds ratio (OR) and 95% confidence intervals (CI) for relative risks (RR) were also calculated for each variation.

Chapter III: Results

6- Results:

6.1- Clinical pathology data:

The individual distributions of the SCA subjects included in the study, according to the clinical pathologic and biological features of biological samples are presented in the table 2 (materials and methods chapter). The distribution of SCA subjects by gender shows the predominance of the disease in male subjects comparing with female individuals (80.4% versus 19.6%). In terms of tumour localization, it was observed a small difference between gender distribution among colon and rectum groups, however, no significant differences were found (table 4). Moreover, 64.7% of the SCA biopsies were located in colon and 35.3% of the SCA biopsies were located in rectum (table 4). There were also no significant differences between mean age in both groups (colon samples: mean age of 73.7 +/- 10 years; rectum samples: mean age of 71.2 +/- 12.1 years).

	Colon		Rectum				
	n	%	n	%	*p*	OR	RR
Male	27	81.8	14	77.8	0.603	1.322 (0.409-4.252)	1.114 (0.755-1.942)
Female	6	18.2	4	22.2			
Total	33	64.7	18	35.3			

Table 4: Gender distribution among colon and rectum groups.

6.2 – Analysis of purity and concentration of DNAs:

Although the concentration of DNA is not uniform (+/-29.3μg/ml) and lower than the standard value (100 μg/ml) (Green et al., 2012) (table 5), the amplification by PCR-SSP occurred without problems, as the protocol of amplification was adapted to the concentration of DNA of samples. Although the mean values of purity of DNAs range between acceptable limits, 1.6-1.8 to O.D.260nm/O.D.280nm (Green et al., 2012) and 0.4-0.6 to O.D.230nm/O.D.260nm (Green et al., 2012), some DNAs show high quantities

of contaminants and proteins with a confidence interval being above certain acceptable limits (table 5).

Furthermore, coefficients of variation show the existence of samples that deviate from acceptable patterns, whether in concentration of DNA and whether in quantity of contaminants. These DNAs can affect some results; however the majority of samples show a level of purity highly acceptable (table 5). Globally, PCR-SSP did not present major amplification problems.

	Concentration	O.D.260nm/O.D.280nm	O.D.230nm/O.D.260nm
Mean	42.4	1.83	0.54
Standard Deviation	29.316	0.09	0.15
Variation Coefficient	69.14%	5%	28%
Confidence Interval (95%)		1.7-1.9	0.4-0.7

Table 5: Means and standard-deviations of DNA samples purity and concentration values.

6.3- Molecular analysis of *MTHFR*, *MTR* and *MTRR* genes:

6.3.1- Polymorphisms in SCA and controls:

Allele frequencies from polymorphisms of *MTHFR*, *MTR* and *MTRR* genes in SCA subjects and controls are presented in table 6. Almost all genes presented a significant difference between both groups, only *MTHFR* had no significant difference among groups (table 6). The allele frequencies A of *MTHFR* 1298 (wild-type) polymorphism are higher in controls than patients (60.8% versus 81%; *p*=0.0001; OR= 0.364; 95% CI 0.206-6.917) (table 6). Whereas the allele C of *MTHFR* 1298 (mutant) polymorphism was more frequent in SCA subjects (39.2% versus 19%; *p*=0.0001; OR= 1.853; 95% CI 1.330-2.504). The allele frequencies A of *MTR* 2756 (wild-type) polymorphism are higher in controls than SCA subjects (44.1% versus 86%; *p*=0.0001; OR= 0.129; 95% CI 0.071-0.261) (table

6). In contrast, the allele G of *MTR* 2756 (mutant) polymorphism was more prevalent in SCA subjects (55.9% versus 14%; *p*=0.0001; OR= 0.564; 95% CI 0.333-0.954).

The allele frequencies A of *MTRR* 66 (wild-type) polymorphism are higher in SCA subjects than controls (66.7% versus 53%; *p*=0.023; OR= 1.774; 95% CI 1.049-3.007) (table 6). On the other hand, the allele G of *MTRR* 66 (mutant) polymorphism was more prevalent in controls (33.3% versus 47%; *p*=0.023; OR= 0.564; 95% CI 0.333-0.954).

Allele Frequencies	SCA and controls						
	SCA		Controls		*p*	OR	RR
	n	%	n	%			
MTHFR C677T							
C	59	57.8	138	69	0.055	0.616 (0.365-1.041)	0.731 (0.529-1.027)
T	43	42.2	62	31		1.878 (1.241-3.140)	1.351 (0.989-2.061)
Total	102	100	200	100			
MTHFR A1298C							
A	62	60.8	162	81	0.0001	0.364 (0.206-6.917)	0.540 (0.399-0.752)
C	40	39.2	38	19		2.750 (1.562-4.849)	1.853 (1.330-2.504)
Total	102	100	200	100			
MTR A2756G							
A	47	44.1	172	86	0.0001	0.129 (0.071-0.261)	0.309 (0.234-0.419)
G	55	55.9	28	14		7.188 (3.971-13.077)	3.088 (2.279-4.071)
Total	102	100	200	100			
MTRR A66G							
A	68	66.7	106	53	0.023	1.774 (1.049-3.007)	1.471 (1.032-2.132)
G	34	33.3	94	47		0.564 (0.333-0.954)	0.680 (0.469-0.969)
Total	102	100	200	100			

Table 6: Allele frequencies distribution among SCA and control subjects.

Genotype frequencies from polymorphisms of *MTHFR*, *MTR* and *MTRR* genes in SCA subjects and controls are presented in table 7. Almost all genotypes from all genes presented a significant difference between both groups, except the genotypes *MTHFR* 677TT, *MTHFR* 1298GG, *MTRR* 66AA and *MTRR* 66AG that had no significant difference among groups (table 7).

	SCA and controls						
Genotypes	SCA		Controls		*p* value	OR	RR
	n	%	n	%			
***MTHFR* C677T**							
CC	12	23.529	48	48	0.004	0.333 (0.145-0.753)	0.467 (0.247-0.827)
CT	35	68.627	42	42	0.002	3.021 (1.401-6.571)	2.102 (1.251-3.650)
TT	4	7.843	10	10	0.667	0.766 (0.190-2.857)	0.833 (0.269-1.804)
Total	51	100	100	100			
***MTHFR* A1298C**							
AA	15	29.411	68	68	0.0001	0.184 (0.082-0.409)	0.331 (0.191-0.559)
AC	32	62.745	26	26	0.0001	4.794 (2.194-10.566)	2.701 (1.660-4.401)
CC	4	7.843	6	6	0.030	0.308 (0.085-1.003)	0.400 (0.126-1.002)
Total	51	100	100	100			
***MTR* A2756G**							
AA	6	11.764	76	76	0.0001	0.042 (0.014-0.119)	0.112 (0.046-0.242)
AG	35	68.627	20	20	0.0001	8.750 (3.806-20.417)	3.818 (2.321-6.312)
GG	10	19.607	4	4	0.002	5.854 (1.562-23.719)	2.387 (1.319-3.221)
Total	51	100	100	100			
***MTRR* A66G**							
AA	22	43.137	28	28	0.062	1.951 (0.908-4.197)	1.532 (0.938-2.420)
AG	24	47.058	49	49	0.822	0.925 (0.445-1.920)	0.950 (0.582-1.541)
GG	5	9.803	23	23	0.049	0.364(0.112-1.105)	0.477 (0.174-1.067)
Total	51	100	100	100			

Table 7: Genotype frequencies distribution among SCA and control subjects.

The genotype *MTHFR* 677CC is significantly higher in controls than SCA subjects (23.529% versus 48%; *p*=0.004; OR= 0.333; 95% CI 0.145-0.753) (table 7). Whereas the genotype *MTHFR* 677CT was more prevalent in SCA subjects (68.627% versus 42%; *p*=0.002; OR= 3.021; 95% CI 1.401-6.571).

The genotype *MTHFR* 1298AA is higher in controls than SCA subjects (29.411% versus 68%; *p*=0.0001; OR= 0.184; 95% CI 0.082-0.409) (table 7). On the other hand, the genotype *MTHFR* 1298AC was more prevalent in SCA subjects (62.745% versus 26%; *p*=0.0001; OR= 4.794; 95% CI 2.194-10.566).

The genotype *MTR* 2756AA is more prevalent in controls than SCA subjects (11.764% versus 76%; *p*=0.0001; OR= 0.042; 95% CI 0.014-0.119) (table 7). In contrast, the genotype *MTR* 2756AG was higher in SCA subjects (68.627% versus 20%; *p*=0.0001; OR= 8.750; 95% CI 3.806-20.417). In addition, the genotype *MTR* 2756GG is more prevalent in SCA subjects than controls (19.607% versus 4%; *p*=0.002; OR= 5.854; 95% CI 1.562-23.719) (table 7). The genotype *MTRR* 66GG is higher in controls than SCA subjects (9.8% versus 23%; *p*=0.049; OR= 0.364; 95% CI 0.112-1.105) (table 7).

6.3.2- Polymorphisms in patients stratified by clinical pathological features:

6.3.2.1- Gender:

The distribution of SCA subjects by gender shows no significant differences between men and women (tables 8 and 9).

Allele Frequencies	Gender						
	Male		Female		*p*	OR	RR
	n	%	n	%			
MTHFR C677T							
C	45	54.9	14	70	0.222	0.521 (0.159-1.646)	0.886 (0.753-1.105)
T	37	45.1	6	30		1.919 (0.608-6.273)	1.128 (0.905-1.328)
Total (102)	82	100	20	100			
MTHFR A1298C							

	n	%	n	%	p	OR	RR
A	48	58.5	14	70	0.349	0.605 (0.185-1.915)	0.911 (0.772-1.142)
C	34	41.5	6	30			
Total (102)	82	100	20	100		1.653 (0.522-5.414)	1.098 (0.876-1.295)
MTR A2756G							
A	36	43.9	11	55	0.374	0.640 (0.215-1.895)	0.916 (0.750-1.128)
G	46	56.1	9	45			
Total (102)	82	100	20	100		1.562 (0.528-4.658)	1.092 (0.886-1.334)
MTRR A66G							
A	55	67.1	13	65	0.861	1.097 (0.347-3.403)	1.019 (0.841-1.307)
G	27	32.9	7	35			
Total (102)	82	100	20	100		0.912 (0.294-2.883)	0.982 (0.765-1.189)

Table 8: Statistical analysis of allele frequencies in gender.

Genotypes	Gender						
	Male		Female		*p*	OR	RR
	n	%	n	%			
MTHFR C677T							
CC	8	19.5	4	40	0.175	0.364 (0.065-2.015)	0.788 (0.465-1.124)
CT	29	70.7	6	60	0.516	1.611 (0.308-8.263)	1.105 (0.831-1.624)
TT	4	9.75	0	0	0.208	inf (0.144-inf)	1.270 (0.507-1.270)
Total (51)	41	100	10	100			
MTHFR A1298C							
AA	11	26.8	4	40	0.417	0.550 (0.106-2.905)	0.880 (0.584-1.184)
AC	26	63.4	6	60	0.843	1.156 (0.225-5.760)	1.029 (0.793-1.441)
CC	4	9.75	0	0	0.308	inf (0.144-inf)	1.270 (0.507-1.270)
Total (51)	41	100	10	100			
MTR A2756G							
AA	4	9.75	2	20	0.372	0.432 (0.051-4.139)	0.811 (0.304-1.194)
AG	28	68.2	7	70	0.918	0.923 (0.157-4998)	0.985 (0.779-1.442)
GG	9	21.9	1	10	0.398	2.531 (0.256-60.441)	1.153 (0.702-1.313)
Total (51)	41	100	10	100			
MTRR A66G							
AA	18	43.9	4	40	0.825	1.174 (0.239-5.965)	1.032 (0.756-1.326)
AG	19	46.3	5	50	0.837	0.864 (0.178-4.185)	0.972 (0.731-1.284)
GG	4	9.75	1	10	0.982	0.973 (0.081-25.783)	0.995 (0.379-1.262)
Total (51)	41	100	10	100			

Table 9: Statistical analysis of genotype frequencies in gender.

6.3.2.2- Tumour localization:

Allele frequencies from polymorphisms of *MTHFR*, *MTR* and *MTRR* genes in SCA subjects and controls are presented in table 10. The majority of genes did not present a significant difference between both groups, only *MTRR* had significant difference among groups (table 10).

Allele frequencies	Tumour localization						
	Colon		Rectal		*p*	OR	RR
	n	%	n	%			
*MTHFR*677							
C	35	53.0	24	66.7	0.185	0.565 (0.222-1.422)	0.823 (0.623-1.135)
T	31	47.0	12	33.3		1.771 (0.703-4.505)	1.215 (0.881-1.605)
TOTAL (102)	66	100	36	100			
*MTHFR*1298							
A	41	62.1	21	58.3	0.709	1.171 (0.471-2,909)	1.058 (0.779-1.491)
C	25	37.9	15	41.7		0.854 (0.344-2.122)	0.945 (0.671-1.284)
TOTAL (102)	66	100	36	100			
*MTR*2756							
A	26	39.4	21	58.3	0.068	0.464 (0.187-1.147)	0.761 (0.553-1.049)
G	40	60.6	15	41.7		2.154 (0.872-5.360)	1.315 (0.953-1.808)
TOTAL (102)	66	100	36	100			
*MTRR*66							
A	39	59.1	29	80.6	0.029	0.349 (0.119-0.992)	0.722 (0.577-0.997)
G	27	40.9	7	19.4		2.868 (1.008-8.422)	1.385 (1.003-1.733)
TOTAL (102)	66	100	36	100			

Table 10: Statistical analyses of allele frequencies in tumour localization.

The allele frequencies A of *MTRR* 66 (wild-type) polymorphism are higher in rectal SCA subjects than colon SCA subjects (59.1% versus 80.6%; *p*=0.029; OR= 0.349; 95% CI 0.119-0.992) (table 10). Whereas the allele G of *MTRR* 66 (mutant) polymorphism was more prevalent in colon SCA subjects (40.9% versus 19.4%; *p*=0.029; OR= 2.868; 95% CI 1.008-8.422).

Genotypes	Tumour localization						
	Colon		Rectal		*p*	OR	RR
	n	%	n	%			
*MTHFR*677							
CC	5	15.1	7	38.9	0.059	0.281 (0.059-1.281)	0.580 (0.230-1.087)
CT	25	75.8	10	55.6	0.141	2.500 (0.626-10.206)	1.429 (0.856-2.760)
TT	3	9.1	1	5.5	0.657	1.700 (0.137-46.032)	1.175 (0.333-1.596)
TOTAL (51)	33	100	18	100			
*MTHFR*1298							
AA	8	24.2	7	38.9	0.277	0.503 (0.122-2.049)	0.768 (0.395-1.256)
AC	25	75.8	7	38.9	0.010	4.911 (1.219-20.761)	1.855 (1.073-3.407)
CC	0	0	4	22.2	0.005	0 (0-0.758)	0 (0-0.900)
TOTAL (51)	33	100	18	100			
*MTR*2756							
AA	0	0	6	33.3	0.0001	0 (0-0.406)	0 (0-0.676)
AG	26	78.9	9	50	0.036	3.714 (0.911-15.721)	1.698 (0.968-3.461)
GG	7	21.2	3	16.7	0.699	1.346 (0.253-7.829)	1.104 (0.539-1.575)
TOTAL (51)	33	100	18	100			
*MTRR*66							
AA	11	33.3	11	61.1	0.058	0.318 (0.081-1.217)	0.659 (0.404-1.071)
AG	17	51.5	7	38.9	0.393	1.670 (0.448-6,333)	1.195 (0.752-1.810)
GG	5	15.1	0	0	0.085	inf (0.478-inf)	1.643 (0.732-1.643)
TOTAL (51)	33	100	18	100			

Table 11: Statistical analyses of genotype frequencies in tumour localization.

Genotype frequencies from polymorphisms of *MTHFR*, *MTR* and *MTRR* genes in colon SCA subjects and rectal SCA subjects are presented in table 11. The majority of

genotypes from all genes did not present a significant difference between both groups, except the genotypes *MTHFR* 1298AC, *MTHFR* 1298CC, *MTR* 66AA and *MTR* 66AG that had significant difference among groups (table 11).

The genotype *MTHFR* 1298AC is higher in colon SCA subjects than rectal SCA subjects (75.8% versus 38.9%; *p*=0.010; OR= 4.911; 95% CI 1.219-20.761) (table 11). On the other hand, the genotype *MTHFR* 1298CC was more prevalent in rectal SCA subjects (0% versus 22.2%; *p*=0.005; OR= 0; 95% CI 0-0.758).

The genotype *MTR* 2756AA is higher in rectal SCA subjects than colon SCA subjects (0% versus 33.3%; *p*=0.0001; OR= 0; 95% CI 0-0.406) (table 11). Whereas, the genotype *MTR* 2756AC was more prevalent in colon SCA subjects (78.9% versus 50%; *p*=0.036; OR=3.714; 95% CI 0.911-15.721).

Chapter IV: Discussion

7- Discussion

The development of SCA is the result of a complex interaction of variables, including external factors such as exposure to environmental agents and dietary factors and internal factors. It has not been established if genotypes with low penetrance polymorphisms in genes related to metabolism of folic acid such as methylenetetrahydrofolate reductase (MTHFR C677T and A1298C), methionine synthase (MTR A2756G) and methionine synthase reductase (MTRR A66G) are associated with the risk of the disease or even its clinical manifestations. Therefore, the aim of this study was to determine whether these genetic polymorphisms influence the risk of sporadic colorectal adenocarcinoma (SCA) and their clinical and biological manifestations.

This study was based upon the assumption that the genotypes of *MTHFR* A1298C, *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G involved in the metabolism of folic acid, alter the risk and the clinical and biological features of the tumour in patients, in spite of the existence of innumerous conflicting evidences about the issue. The enzymes codified by the genes under study can present significant alteration in their function due to different combinations between wild-type and variant alleles, subsequently leading to abnormalities in synthesis of DNA, reparation machinery or during the methylation process. These changes consequently lead to the alteration in the risk of malignant diseases, whether conferring a certain protection, whether increasing the risk of development of the disease.

On the other hand, the deficiency of folate can also affect enzymatic activities and alter the risk of cancer by two mechanisms: hypomethylation of DNA activating a proto-oncogene and false incorporation of uracil in DNA. As it is referred before, these mechanisms are also a result from the impact of variant alleles.

7.1- SCA subjects and controls:

Folate and folate metabolism genes seem to be responsible in the pathogenesis of colon and rectal cancer, as they are involved in DNA maintenance, repair and methylation. In

addition, recent research suggests that *MTHFR, MTR* and *MTRR* are key genes in the influence of risk in SCA. This hypothesis is corroborated with the results of this work, since it was found high correlation in mutant alleles, such as C from polymorphism *MTHFR* A1298C and G from polymorphism *MTR* A2756G, as well as the wild-type allele A from polymorphism MTRR 66. The study also showed that genotypes *MTHFR* 677CT, *MTHFR* 1298 AG, *MTR* 2756AG and *MTR* 2756GG were associated to risk in SCA.

MTHFR is a key enzyme because it generates 5-MTHF, which is the active form of folate in plasma (Choi et al., 2002). The 5-MTHF is the essential methyl group donor involved in methylation processes. Thus, genetic changes in the *MTHFR* gene can influence the risk of SCA (Shrubsole et al., 2006; Duthie et al., 2011). Heterozygous carriers in this study showed association with SCA, which can be explained by a relatively lower enzyme activity (65%) (Frosst et al., 1995). This genotype is also associated with higher plasma homocysteine and reduced plasma folate levels, (Frosst et al., 1995) leading to genomic DNA hypomethylation (Ulrich et al., 2005) concomitant with gene-specific promoter hypermetylation (Oyama et al., 2004), as it is frequently observed in cancer.

There seems to be statistically significant associations between CRC risk and *MTHFR* C677T polymorphisms, since it expresses a rate-limiting in the methyl cycle.

Although the variant homozygous genotype did not show significant association with SCA in this work, other studies observed that the risk was 2,42 times higher to CRC in individuals for this genotype (Cao et al., 2008). Iacopetta et al., showed that individuals with variant T allele and deficiency of folate presented a higher risk of CRC in proximal colon (Iacopetta et al., 2009). Other studies found an association of higher risk of CRC in patients who carried the wild-type C allele (Wang et al., 2006; Lima et al., 2007; Chang et al., 2007). In other studies, the gene polymorphism was not associated to the risk of CRC (Keku et al., 2002; Plaschke et al., 2003). Contradictory results in different studies can be due to differences of eating habits, racial variations and lifestyles, besides the fact that eventually other polymorphisms can be present in the process of carcinogenesis. The results of this study showed association between the genotype *MTHFR* 1298 AC and SCA, possibly because the polymorphism *MTHFR* A1298C may result in a reduction of 40% in enzyme activity. In addition, there been reported statistically significant associations between CRC risk and *MTHFR* A1298C

polymorphisms in several studies. In contrast, two metanalyses suggest that the variant homozygous genotype *MTHFR* 1298CC variant protects individuals from CRC (Kono and Chen, 2005; Huang et al., 2007). In this work, the mutated C allele of polymorphism *MTHFR* A1298C is associated to SCA; however, the functional effect of variant C allele is not well comprehended (Izmirli et al., 2013). Both mutated *MTHFR* gene profiles were reported in some cancer types (Izmirli et al., 2011; Izmirli et al., 2013).

There are conflicting results that range from strong linkage to no association between *MTHFR* and CRC risk in different literature findings (Izmirli et al., 2013). Conflicting results in different studies can be explained to differences of diet habits, racial variations and lifestyles, besides the fact that eventually other polymorphisms can be present in the process of carcinogenesis. Therefore, the balance between DNA synthesis and DNA methylation determined by the *MTHFR* polymorphisms may influence the risk of SCA, which is also influenced by folate intake. Thus, more studies or large case-control studies are required, in order to unveil a better understanding of the possible roles of *MTHFR* C677T and A1298C in CRC.

MTR is an important enzyme for the provision of SAM, as well as the provision of THF for use in nucleotide synthesis (Banerjee et al., 1990). Its role is also important in the maintenance intracellular folate homeostasis, avoiding toxic levels of homocysteine (Van Der Put et al., 1997). In this work, it was found that the variant allele G of the polymorphism *MTR* A2756G is associated to the risk of SCA. Recent analyses of the Nurses Health Study and Health Professionals Follow-up Study found a nonsignificant increased risk of CRC for this variant allele (Koushik et al., 2006). Also, this work showed association between the genotypes *MTR* 2756 AG and *MTR* 2756 GG, which can be explained by the significant low levels of the enzyme resulting in accumulation of 5-MTHF and deficiency of intracellular folate derivatives including 5,10-MTHF required for thymidylate biosynthesis, leading to deoxynucleotide pool imbalances (Manson et al., 2007; Ayala et al., 2009; Black et al., 1997). This leads to the accumulation of deoxyuridylate in DNA, possibly leading to strand breaks commonly seen in CRCs (Visser et al., 2012). These results can be explained by the fact that the polymorphism *MTR* A2756G is associated with lower enzyme activity, leading to homocysteine elevation and

DNA hypomethylation (Lamprecht et al., 1996; Lifshitz et al., 1998). The polymorphism *MTR* A2756G probably leads an inadequate oxidization of the cofactor (cobalamine-vitamin B12), with reduction of activity of MTR and consequently increased cellular Hcy levels (Chen et al., 1997). In addition, other studies show that tumours occurring in the *MTR* 2756GG genotype present a low number of hypermethylated CpG islands of tumour-suppressor genes. Thus this genotype has been associated with an increased risk of CRC, as also confirmed by other studies (De Vogel et al., 2009). On the other hand, other studies report a higher enzyme activity in the mutated variants (Lifshitz et al., 1998). One study showed a small reduction of CRC to the genotype *MTR* 2756GG (Ma et al., 1999). Another study did not find any global effect, however inverse association between the genotype *MTR* 2756GG and CRC was found (Le Marchand et al., 2002). Therefore, the influence of the polymorphism *MTR* A2756G in the risk of SCA is still uncertain.

MTRR is responsible for the regulation of MTR. After a period of being activated, MTR turns off (becomes inactive) and MTRR is needed to reactivate MTR in order to continue to produce methionine. The results of this work show that the genotype *MTRR* 66GG is associated to the risk of SCA and it can be explained by the fact that the polymorphism *MTRR* A66G codes a variant protein exhibiting a 4-fold lower activity compared with the wild-type protein in vivo (Olteanu et al., 2002). Mastuo et al. suggested the same results (Matsuo et al., 2002). Thus, the *MTRR* 66G allele decreases the availability of SAM by reducing the level of active MTR. Consequently, DNA hypomethilation may be induced. It has also been reported that *MTRR* 66GG genotype is inversely associated with higher plasma homocysteine levels, due to possible disturbances of the catalytic activity of the protein (Leclerc et al., 1998; Gaughan et al, 2001). On the other hand, other studies show that mutant heterozygous individuals for the *MTRR* A66G polymorphism present a reduced risk of CRC (Le Marchand et al., 2002). In another study, the genotype *MTRR* A66G was not associated to CRC when three ethnic groups were assessed together. However when the group of Caucasians was studied alone, a very close trend to the risk of CRC with increased the number of variant alleles was observed (OR para GG versus AA=1.9; 95 % IC: 1.0-3.8 p=0.07) (Le Marchand et al., 2002).

7.2- Gender:

There appeared to be no link between any of the polymorphic variants and gender, because no significant differences according to genotype distributions in patients could be found. These results can be possibly explained by the fact that the majority of our samples is composed by male subjects.

7.3- Distinction between colonic and rectal Adenocarcinomas:

Colon cells are characterized by a rapid turnover with high rates of DNA synthesis. Several studies have shown a link between low folate status and indicators for DNA damage (Blount et al., 1997; Ames et al., 1999), and the impact of dietary folate intake in reducing the risk of colon cancer (Le et al., 2002; Kono et al., 2005). Rectal carcinomas show similar development mechanisms as in distal colon tumours (descending and sigmoid colon). Therefore, significant differences between rectal and colon carcinomas with respect to genetic features have been reported (Frattini et al. 2004).

The SCA subjects in this study were stratified according to the place of primary tumour: colonic or rectal. The results in this work showed that for the polymorphism *MTHFR* A1298C the genotype *MTHFR* 1298AC was associated to colon tumour, whereas the genotype *MTHFR* 1298CC was more prevalent in rectal carcinoma. Our results are in accordance with the majority of studies that reported that the *MTHFR* 1298CC genotype is associated with reduced risk of colon cancer (Le Marchand et al., 2005). In addition, there are studies that reported an inverse association for the *MTHFR* 1298CC genotype and colon cancer among Caucasians (Keku et al., 2002).

The results from this study showed that in the polymorphism *MTR* A2756G, the genotype *MTR* 2756AA was associated with rectal tumour, while the genotype *MTR* 2756AG was linked to colon carcinoma. The difference between this study and the others can reflect physiological differences in the effects of folate metabolism in the colon versus the rectum. Also, it is important to consider that studies report that associations between some folate-metabolizing enzyme genes and colon cancer are

observed only in the context of folate or alcohol intake (Larsson et al., 2005). This work reinforces that there are some susceptibility differences according to the tumour primary localization.

7.4- <u>Study Limitations:</u>

There may be several possible mechanisms underlying all association studies, such as, the results from interaction of both environmental and genetic factors, which can be responsible for analysis default. The sample size of the present study (51 SCA and 100 controls) may not be large enough to detect the small effect of low penetrance mutations. The combined effect of multiple genes/mutations can provide more reliable information for genetic contribution to risk of SCA. We cannot completely exclude the effects of the other conditions (i.e. weight, gender, diet type, etc.) and residual confounding attributable to the measurement error (namely, unicentric characters, lack of assess of diet in takes, etc…). It is essential a large approach study with large sample size to confirm our outcomes. Still, the present study provides preliminary evidence that *MTHFR*, *MTR* and *MTRR* variants, may contribute to the risk of SCA in Portuguese Population, and may be useful tools in the study of this multifactorial disorder.

Chapter V: Conclusion

8- Conclusion:

The observations and results found in this study show that polymorphisms from *MTHFR*, *MTR* and *MTRR* genes were associated with increased risk of development of SCA. In fact, MTHFR, MTR and MTRR enzymes are reported as essential to the methylation process in the colon and rectum tissue.

The deficiency of folate can affect enzyme activities responsible for reparation, synthesis and methylation of DNA. Two main mechanisms explain how this depletion can affect the risk of CRC: by hypomethylation of DNA and activation of proto-oncogenes or inducing a damaging incorporation of uracil leading to DNA breakage and chromosomal damage.

Therefore, it is supposed that the variant alleles from *MTHFR*, *MTR* and *MTRR* genes that code enzymes dependent on folate can be associated with an increased risk of SCA. In this study it was also highlighted that the primary localization of the tumour may be conditioned by the polymorphisms *MTHFR*, *MTR* and *MTRR*.

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