Universidade de Aveiro Departamento de Biologia
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Patrícia Alexandra Sousa Jegundo

Polimorfismos de genes com atividade antioxidante e desintoxicante na predisposição para o Cancro Colo-retal

Antioxidant and detoxify genes polymorphisms in colorectal cancer predisposition

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Lina Carvalho, Professora Associada com Agregação do Instituto de Anatomia Patológica da Faculdade de Medicina da Universidade de Coimbra e da Mestre Sandra Balseiro, Professora Assistente da Escola Superior e Saúde Dr. Lopes Dias, Instituto Politécnico Castelo Branco e da Professora Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro.

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CIMAGO

GAI
GABINETE DE APOIO À INVESTIGAÇÃO
FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

Dedico este trabalho aos profissionais de saúde, pacientes e famílias que se deparam com a luta contra o cancro no seu dia-a-dia, bem como, aos que de algum modo deram o seu contributo para esta batalha.

"You may have to fight a battle more than once to win it."

Margaret Thatcher

O júri

Presidente

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Palavras-chave

Adenocarcinamo colo-retal esporádico; Superóxido Dismutases; Glutationas S-Transferases; Polimorfismos comuns; PCR.

Resumo

O cancro colo-retal (CCR) resulta de um conjunto gradual de alterações histológicas e genéticas, que se traduz numa proliferação celular descontrolada. Embora existam causas genéticas para a origem deste carcinoma, a maioria dos autores assume causas multifactoriais para a génese do CCR. Das causas não genéticas, a baixa atividade física, uma dieta rica em gorduras e pobre em fibras, bem como, os hábitos tabágicos parecem ter um papel preponderante no desenvolvimento desta patologia. Do mesmo modo, tem vindo a ser descrito que diferentes níveis de stresse oxidativo podem influenciar o desenvolvimento deste tipo de cancro. Desta forma, a manutenção celular do estado oxidação-redução parece ser crucial para a conservação da função dos tecidos e prevenção da carcinogénese. Variações genotípicas nos genes envolvidos neste processo, tais como, MNSOD, SOD3, GSTP1, GSTT1 e GSTM1, podem ser importantes biomarcadores para o CCR.

Neste trabalho pretendeu-se determinar a distribuição da frequência dos polimorfismos mais comuns dos genes envolvidos na regulação do stresse oxidativo (*MNSOD, SOD3, GSTP1, GSTT1* e *GSTM1*) em indivíduos com adenocarcinoma colo-retal esporádico (ACE) e em controlos saudáveis, avaliando assim a sua possível correlação com o risco para o desenvolvimento do ACE. A análise dos polimorfismos dos genes com atividade desintoxicante e desintoxicante (*MNSOD T175C, SOD3 R213G, GSTP1 A105G, GSTP1 C114T, GSTT1del* e *GSTM1del*) foi feita através da técnica de PCR-SSP.

Neste estudo encontrámos uma prevalência dos genes mutados nos pacientes com ACE, comparativamente com o grupo controlo: *MNSOD 175CC* (55% vs 2%; p<0,0001; OR: 58,5; CI 13,3 a 256,7), *SOD3 213GG* (31% vs 2%; p<0,0001; OR: 21,89; CI 4,93 a 97,29), *GSTP1 105GG* (46% vs 12%; p<0,0001; OR: 6,14; CI 2,85 a 13,26), *GSTP1 114TT* 38% vs 0%; p<0,0001; OR: Infinito) e *GSTT1 del* (75% vs 28%; p<0,0001; OR: 7,71; CI 3,83 a 15,56). Além disto, observámos também que os genótipos mutados *GSTP1 114TT* (52% vs 27%; p=0,003; OR: 2,88; CI: 1,41 a 5,89) e *GSTT1 del* (87% vs 65%; p=0.003; OR: 3.66; CI 1.51 a 8.84) estavam associados com o colon.

Deste modo, os nossos resultados sugerem uma associação positiva entre os polimorfismos dos genes estudados e a prevalência do ACE. Assim sendo, a desregulação dos genes *MNSOD, SOD3, GSTP1, GSTT1* e *GSTM1* pode ser associada com um aumento de ROS no tecido do colon-retal. Além disto, o stresse oxidativo nas células do tecido colon-retal pode também induzir uma desregulação da via da p53. Este estudo evidência assim que os polimorfismos *MNSOD 175C, SOD3 213G, GSTP1 105G, GSTP1 114T* e *GSTT1 del* poderão estar envolvidos no risco para o ACE, permitindo clarificar esta patologia multifactorial.

Keywords Sporadic coloretal adenocarcinoma; Superoxide dismutases; Glutatione S-transferases; common Polymorfisms; PCR.

Abstract

Colorectal cancer (CRC) results from histologic and gene alterations can lead to a massive cellular proliferation. Most of the authors assume multifactorial causes to CRC genesis. Low physical activity, a fat diet poor in fibers and smoking habits seems to have an important role in CRC. However, there are also genetic causes associated with CRC risk. It has been described that oxidative stress levels could influence CRC development. Thus, cellular balance reactive species and defense enzymes involved in oxidative stress are crucial to maintain a good tissue function and avoid neoplasic process. Therefore, genome variations on these defense enzymes, such as MNSOD, SOD3, GSTP1, GSTT1 and GSTM1, could be important biomarkers to colorectal adenocarcinomas.

We intend to determine frequencies distribution of most common polymorphisms involved on oxidative stress regulation (*MNSOD, SOD3, GSTP1, GSTT1* and *GSTM1*) in patients with sporadic colorectal adenocarcinoma (SCA) and in healthy controls, evaluation their possible correlation with SCA risk. Samples common polymorphisms of antioxidant and detoxify genes (*MNSOD T175C, SOD3 R213G, GSTP1 A105G, GSTP1 C114T, GSTT1del* and *GSTM1del*) analysis was done by PCR-SSP techniques.

In this study we found a higher prevalence of *MNSOD 175CC* (55% vs 2%; p<0.0001; OR: 58.5; CI 13.3 to 256.7), *SOD3 213GG* (31% vs 2%; p<0.0001; OR: 21.89; CI 4.93 to 97.29), *GSTP1 105GG* (46% vs 12%; p<0.0001; OR: 6.14; CI 2.85 to 13.26), *GSTP1 114TT* (38% vs 0%; p<0.0001; OR: Infinity) and *GSTT1 null* (75% vs 28%; p<0.0001; OR: 7.71; CI 3.83 to 15.56) mutated genotypes among SCA patients, while the normal genotypes were associated with SCA absence. Furthermore, we found *GSTP1 114TT* mutated genotype (52% vs 27%; p=0.003; OR: 2.88; CI: 1.41 to 5.89) and *GSTT1 null* genotype (87% vs 65%; p=0.003; OR: 3.66; CI 1.51 to 8.84) associated with colon samples.

These findings suggest a positive association between most of common polymorphisms involved on oxidative stress regulation and SCA prevalence. Dysregulation of *MNSOD, SOD3, GSTP1, GSTT1* and *GSTM1* genes could be associated with an increase of ROS in colon and rectum tissue and p53 pathway deregulation, induced by oxidative stress on colonic and rectal cells. The present study also provides preliminary evidence that *MNSOD 175C, SOD3 213G, GSTP1 105G, GSTP1 114T* and *GSTT1* null polymorphisms, may be involved in SCA risk and could be useful to clarify this multifactorial disorder.

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List of abbreviations

Ala – Alanine
Arg – Arginine
CAT – Catalase
CI – Confidence Interval
CRC – Colorectal cancer
ECM – Extracellular matrix
EC-SOD – Extracellular superoxide dismutase
FAP – Familial adenomatous polyposis
Gly – Glycine
GSH – Glutathione
GSH-Px – Glutathione peroxidase
GSSRG-R – Glutathione reductase
GST – Glutathione S-transferase
H_2O_2 – hydrogen peroxide
HNPCC - Hereditary nonpolyposis colon cancer
HO^- – hydroxyl radical
Ile – Isoleucine
MAP – MYH-associated polyposis
MnSOD – Manganese dependent superoxide dismutase
NADPH – Nicotinamide adenine dinucleotide phosphate
O_2 – Molecular oxygen
O_2^- – Superoxide radical/anion,
OD – Optical Density
OR – Odd Ratio
PCR-SSP – Polymerase Chain Reaction - Single Specific Primers
RNS – reactive nitrogen species
ROS – reactive oxygen species
RR – Relative Risk
SCA – Sporadic Colorectal Adenocarcinoma
SNP – Small nucleotide polymorphism
SOD – Superoxide dismutase
Val – Valine
WHO – World Health Organization

I. Introduction

1.1. Colorectal Cancer

Cancer is characterized by uncontrolled growth and spread of mutated cells (Figure 1). External factors (such as alcohol and radiation), as well, internal factors (such as hormones and mutations) may trigger initiation and promotion of carcinogenesis.[1-5] When tumor 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. 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.[1-5] Studying the impact of nutrients on genes, their encoded proteins and the influence of genetic factors on diet is essential for the development of strategies of prevention.

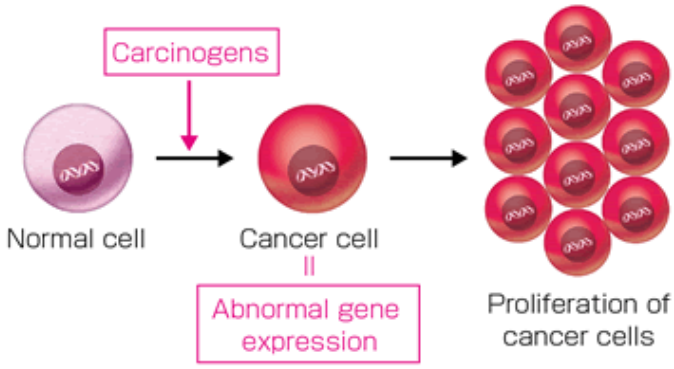


Figure 1: Carcinogenesis process.
Adapted from: http://csls-text2.c.u-tokyo.ac.jp/large_fig/fig07_01.html

Colorectal cancer (CRC) is the second major cause of death on the developed countries and the second more incident in both genders (Figure 2).[6] The incidence of CRC patients and their overall survival change between countries, ethnic groups and lifestyles.[1-4] The interaction between diet and other environmental factors with genetic factors can explain the difference in incidence between geographic regions.[1-4] Mortality by CRC has increased approximately

80% during the last two decades of the 20th century.[5] Based on statistics from data of 2012, CRC is responsible for 13% of death by in Europe and its 16% of the total oncologic mortality rate in Portugal. In Portugal CRC have the 2nd higher mortality after lung in males and breast cancer in females (Figure 3).[6]

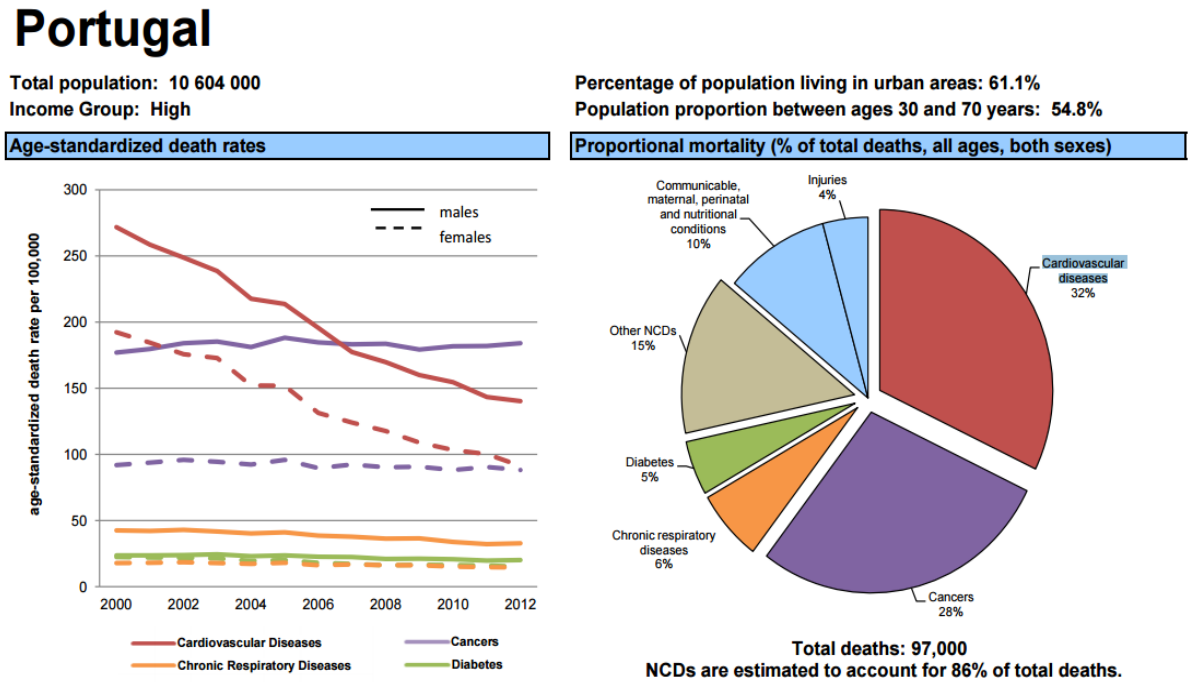


Figure 2: Cancer incidence in Portugal by World Health Organization (2014). Adapted from: http://www.who.int/nmh/countries/prt_en.pdf?ua=1.

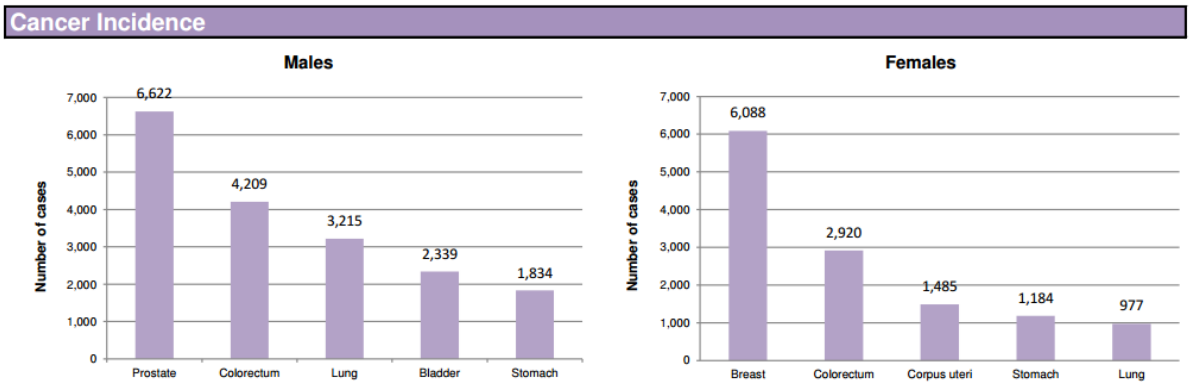


Figure 3: CRC incidence in males and females in Portugal by World Health Organization (2014). Adapted from: http://www.who.int/nmh/countries/prt_en.pdf

1.2. Types of Colorectal Cancer

There are three known types of this disease: sporadic forms, inherited syndromes and family forms.[7,8] Approximately 10%-30% of all CRC cases occur in the context of a family history (such as Lynch Syndrome) 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. [9-11] These cases result from mutations highly associated to specific genes that lead to the development of two distinct syndromes: familial adenomatous polyposis (germinal mutation in *APC* gene) and non-associated polyposis colon inherited carcinoma (Syndrome of Lynch - germinal mutations in genes involved in DNA repair).[12-14] The accumulation of these mutations results from an inactivated repair system, and this is defined as the mutant pathway of carcinogenesis.[15] 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%.[16] In spite of the familial cases, approximately 70% of CRC cases occur occasionally, indicating that health behaviors are strongly correlated to disease development. [17,18]

Sporadic form of CRC (Sporadic Colorectal Carcinoma - SCA) is characterized by accumulation of mutations, loss of function of several tumor suppressor genes and activation of oncogenes. [19] These oncogenes are involved in regulation of cell proliferation and tumor suppressor genes. Gain-of function of oncogenes and loss-of-function mutations in tumor suppressor lead to altered cell proliferation.[20,21] In SCA, random mutations during carcinogenesis follow a well-

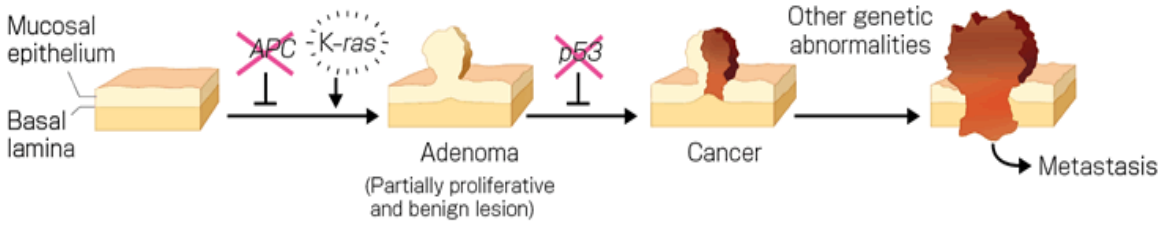


Figure 4: Multistep model of Colorectal carcinogenesis.
Adapted from: http://csls-text2.c.u-tokyo.ac.jp/large_fig/fig07_04.html

established sequence (Figure 4). The inactivation of *APC* gene located in chromosome 5 is the first step to dysplasia in an adenoma. Additional mutations accumulate in oncogenes (*RAS* family) and tumor suppressor genes in chromosomes 18q (*DCC*, *SMAD2*, *SMAD4*) and 17q (*p53*), leading to an accentuated dysplasia and subsequently to carcinoma.[22]

It is also know that colitis associated to CRC has a contribution to carcinogenesis. The mutation and transformation process of a normal into a cancer cell can be triggered by accumulation of free radicals at the early stages and result in cancer progression. Generally is a slow process and often takes decades from tumor initiation to diagnosis.[23]

1.3. Environmental factors: antioxidant nutrients

Diet and environment have been recognized as main factors to CRC development risk. It is known that low physical activity, a rich diet in fats and sugars and poor in food fibers, as well smoking habits were associated with a greater CRC risk. On the other hand, high consumption of vegetables and an active life have been associated with a low risk. [24-27] Various nutrients with either pro-inflammatory or anti-inflammatory activities may work together to influence CRC risk. High consumption of processed and red meats, refined grains, soda, and sweets pattern has been associated with increased CRC risk. In contrast, high intake of fruits, vegetables, fish, poultry, and whole-grain products has been associated with lower risk. Glutathione, carotenoids (β-carotene, vitamin A and lycopene), vitamin C, vitamin E and flavonoids have potent anti-oxidative and anti-inflammatory properties, which confers protective effects against oxidative stress and tumorigenesis. Prospective studies have shown that antioxidant nutrient intake is associated inversely with CRC risk and can reduce adenoma recurrence. However, antioxidant intake may be essential for overall health, but is unlikely to prevent CRC. [27-32]

Among CRC patients, higher intake of a Western dietary pattern after diagnosis may increase the risk of cancer recurrence and mortality, while a Mediterranean diet has been associated with lower cancer mortality and lower CRC risk.[27] Despite of the consistent associations between CRC and some food behaviors (diet, smoking habits, physical activity), there are several inconsistencies which suggest that genes variants (polymorphisms) are involved on body individual responses to their behavior. These inconsistencies can reflect the complex and multifactorial genesis of this pathology (Figure 5). Furthermore, polymorphisms can interfere with bioactive process, absorption and elimination. Molecular targets and their distribution can be altered, as well. In this view, at first, it is essential understand the interaction between those factors and their role in CRC development. [33-36]

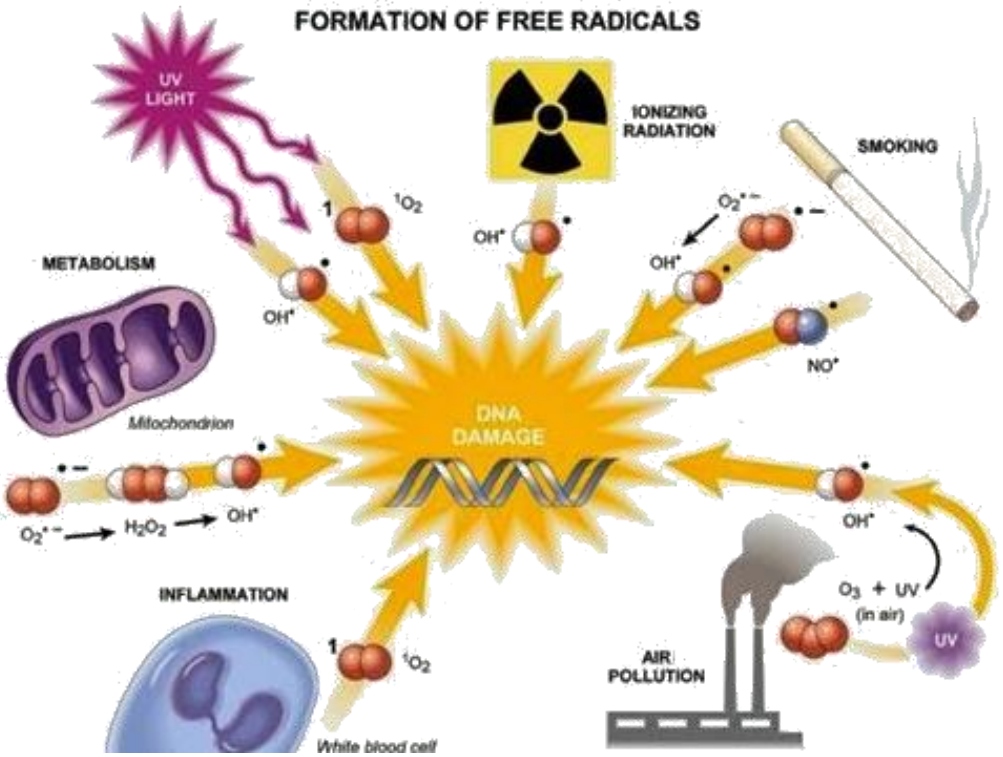


Figure 5: Main sources of ROS production.
Adapted from: http://transformationalwellnessproject.blogspot.pt/2014/03/topic-2-reduction-oxidation-free.html

Inflammation, fat metabolism, meat consumption, alcohol and tobacco smoking are the main factors in CRC genesis by reactive oxygen species (ROS) release (Figure 6). Activated macrophages during inflammation, oxidation of polyunsaturated fatty acids, intake of heme present in red meat release large amounts of ROS and reactive nitrogen species (RNS) witch oxidize DNA/RNA, proteins and lipids and induce DNA damage and cell proliferation of colonic epithelial cells. High levels of insulin and glucose, in combination with oxidative stress and chronic inflammation, can increase the risk of developing cancer amongst patients with diabetes.[28] Although controversial, epidemiologic studies of tobacco smoking have demonstrated high levels of oxidative damaged in the leukocytes of lung cancer patients. However the molecular mechanism of development of CRC remains unclear.[29]

Adult Risk Factors

	Males	Females	Total
Current tobacco smoking (2011)	30.1%	14.8%	22.1%
Total alcohol per capita consumption, in litres of pure alcohol (2010)	18.7	7.6	12.9
Physical inactivity (2010)	33.5%	40.8%	37.3%
Obesity (2014)	21.4%	22.8%	22.1%
Household solid fuel use (2012)	-	-	0.0%

Figure 6: Risk factors to CRC carcinogenesis.
Adapted from: http://www.who.int/nmh/countries/prt_en.pdf

1.4. Oxidative stress

There are several metabolisms involved in cancer beginning. From those, the redox metabolism has been one of the most studied on cancer research. Redox homeostasis is maintained by regulated production of redox active molecules. Imbalance on this metabolism leads to an overproduction of ROS, increasing the rates of cellular oxidative stress.[28-30, 37-42] ROS are molecules or ions formed by the incomplete one-electron reduction of oxygen. Mitochondria are essential for the production of energy inside the cell. This is the most important

source of ROS. They are unstable metabolites of molecular oxygen (O_2) that have high reactivity, such as, superoxide radical (O_2^-), hydroxyl radical (HO^-) and hydrogen peroxide (H_2O_2). These species are generated as byproduct of normal aerobic metabolism. In balance conditions, they contribute to microbicide activity of phagocytes, regulation of signal transduction and gene expression. [32,43,44]

Oxidative stress is defined as overproduction of reactive oxygen species combined with failure in protective mechanisms of redox defense. The concentrations (threshold), pulse duration (flux) and sub-cellular localization are responsible for their responses (Figure 7).[32,44,45] Low or moderate concentrations, reactive oxygen species act as mediators/second messengers of specific physiological processes and signaling pathways regulating numerous cellular processes, including proliferation and apoptosis. However, ROS and RNS high concentrations can be responsible for: damage on DNA, RNA, proteins and chromosome degradation; polyunsaturated fatty acids and amino acids oxidation; enzymatic inactivation, abnormal inflammatory reactions and interfere on cell-signaling molecule, such as apoptotic regulators and antioxidant enzymes. Those alterations seem to be associated with the initiation or progression of human cancers by the disruption of cell, tissue or organ functions (Figure 8). [28, 29, 41, 42, 44, 46-51]

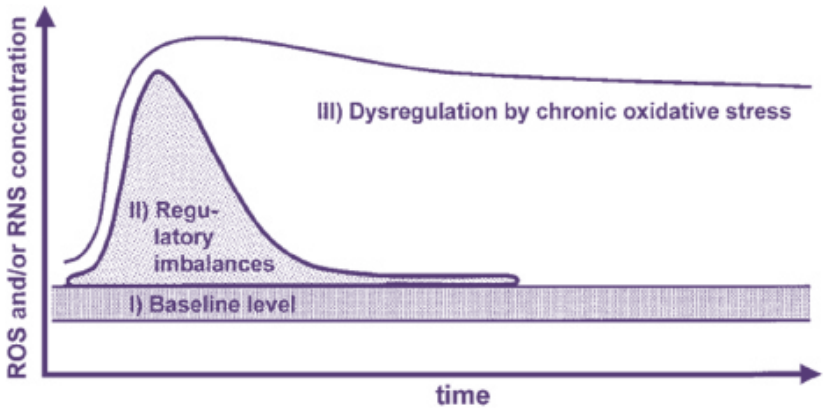


Figure 7: Reactive species concentration and their responses.
Adapted from: Graves DB. *The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology*. J Phys D Appl Phys. 2015; 45: 263001

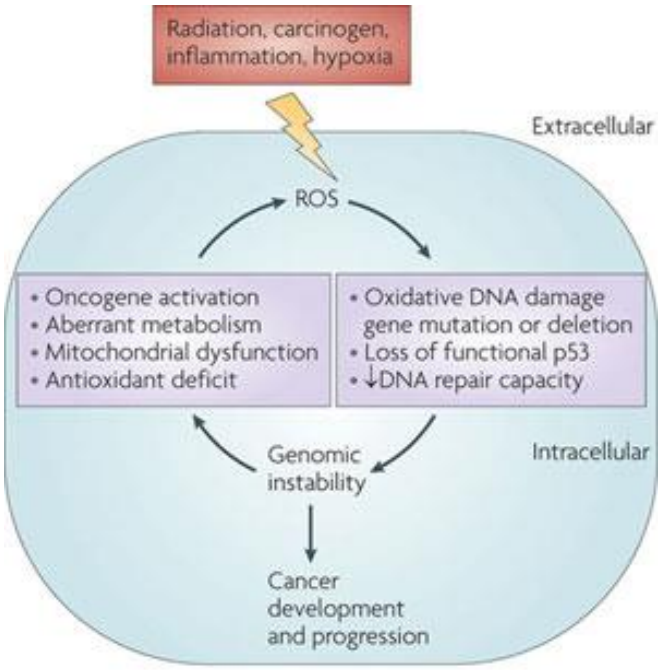


Figure 8: ROS-mediated mechanisms in carcinogenesis.
Adapted from: Trachootham D. Alexandre J. Huang P. *Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?* Nature Reviews Drug Discovery.2009: 8, 579-591

Free radicals are molecules with high instability and reactivity due to the presence of an odd number of electrons in the outermost orbit of their atoms. Cells can generate ROS from exogenous sources as well as endogenously. Endogenously ROS production results mainly from the mitochondrial electron transport chain but also from proteins metabolism (metabolism of fatty acid, xanthine oxidases, cytochrome P450 reductase, nitric oxide synthase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, peroxisomes and myeloperoxidase.[23, 41, 44, 51, 52] Molecular oxygen is the last electron acceptor during oxidative phosphorylation. Incomplete reduction of O_2 in the mitochondrial electron transport chain can lead to accumulation of O_2^-, OH^- and H_2O_2, which as highly reactive species.[30,43]

1.5. Genetic Factors: Oxidative stress regulators

Nowadays, we know that human gene sequence is shared for 99,9% of individuals and the slight variation is essentially in just one nucleotide (SNPs - small nucleotide polymorphisms). [87] Polymorphism is variation in a gene or in DNA that occurs with a relative high frequency on population. The recent medicine attempts to relate those polymorphisms with the individual response to diet behavior, tobacco and alcohol consumption with disease predisposition. Noting that individual genetic constitution alters individual response to bioactive components.[38, 39, 53-59] On this view, in last years it has been reported several variations, directly involved or not, involved in metabolic pathways with direct risk to CRC development.[25-27, 33-36, 60-63]

Eukaryotic cells have developed defense mechanisms that eliminate ROS. The human's antioxidant endogenous defense system consists in a range of extracellular and intracellular antioxidants that are able to protect tissues from ROS and RNS. This antioxidant defense consists in non-enzymatic and enzymatic system. Non-enzymatic antioxidants are nutrient compounds, which includes: fenol, glutathione, vitamins C and E, β-carotene, α-tocopherol, and cytochrome c. Enzymatic process includes antioxidant enzymes, such as superoxide dismutase (SOD), glutathione reductase (GSSRG-R), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), and catalase (CAT). SOD catalyzes the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) plus O_2. GSH-Px and catalase reduce H_2O_2 to O_2 and H_2O (Figure 9). GSH-Px uses glutathione as a reducing agent (electron donor). [23, 32, 41, 42, 45, 47, 48, 51, 64-68, 69, 70]

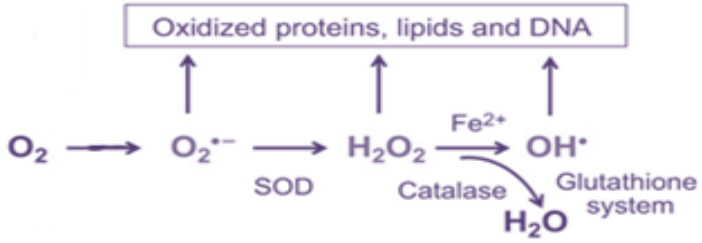


Figure 9: ROS elimination process.
Adapted from: Bigarella CL. Liang R. Ghaffari S.. Stem cells and the impact of ROS signaling. Development. 2014; 141(22): 4206–4218.

Some genes have polymorphic variants, single nucleotide polymorphisms (SNP) that are responsible for the individual's genetic constitution/response. Between the genes with antioxidant and detoxifying activity, superoxide dismutases (SOD) and glutathione-*S*-transferases (GST) genes are highly polymorphic and often contain numerous mutations, increasing the risk of the cancer development on esophagus, stomach, large intestine, breasts, lungs and lymph nodes.[39, 48, 53-56] Thus, we can suppose that some polymorphisms, involved in oxidative stress regulation, may predispose to disease and particularly on CRC. The strength of the biological impact will also depend on heterozygosity or homozygosity of the variant allele.[71]

1.5.1. Antioxidant regulators - Superoxide Dismutases

It is assumed that carcinogenesis is a result of cellular proliferation and differentiation control loss. Superoxide dismutase genes seem to have an important role on oxidative metabolism balance, particularly in cellular detoxify. SODs are a family of enzymes, responsible for the first line of antioxidant defense against ROS.[67,72] In humans there are three main isoforms of these genes: *SOD1, SOD2* and *SOD3* (Figure 10). They encode proteins that require copper, zinc or manganese on their active center in order to their enzymatic function, respectively SOD1, SOD3 (Cu/Zn- SOD) and SOD2. O_2^- dismutation mechanism into H_2O_2 by SOD, which involves alternate reduction and re-oxidation of these metals.[73] SOD1 is located in the cytosol of liver, kidney, erythrocytes, and central nervous system cells – mutations on this enzyme are associated with neural diseases, such as amyotrophic lateral sclerosis. SOD2 is found in mitochondria and it's associated with aging, cancer, asthma and transplant rejection as well. SOD3 is detected as extracellular enzyme, plays an important role in regulating blood pressure, vascular contraction and also in neurologic, pulmonary and arthritic diseases. SODs catalyze the inactivation (dismutation) of superoxide ion by convert in oxygen peroxide, a less toxic molecule. [23, 38, 39, 43, 56, 64, 74, 75]

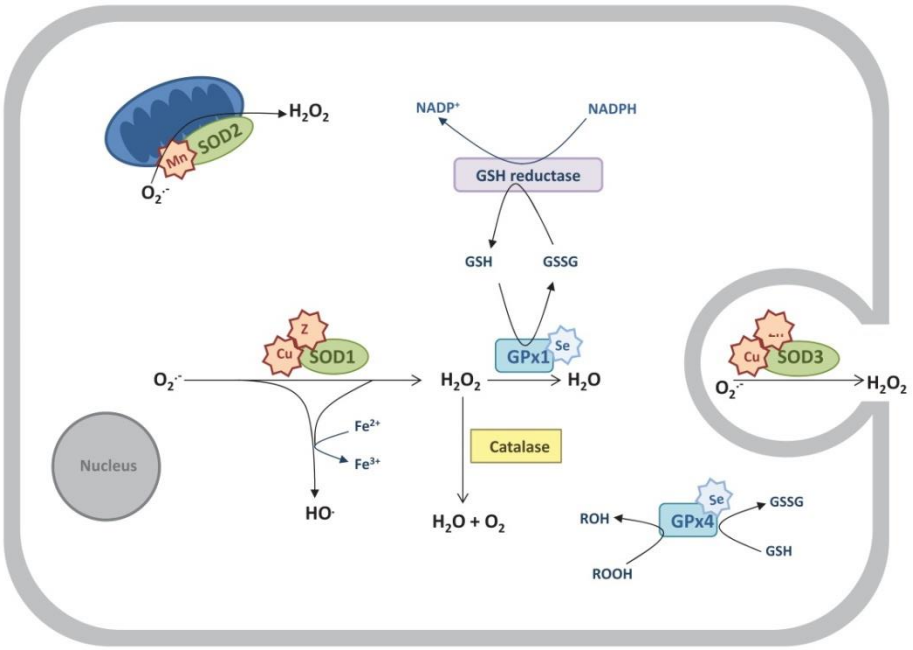


Figure 10: Role of SODs in oxidative metabolism balance.
Adapted from: http://cdn.intechopen.com/pdfs-wm/38459.pdf

There is evidence that decreased of intracellular SOD levels are related to malignant transformation, nevertheless, this data was not reported for SOD1.[67, 72, 76] In the past, it was supposed that induction higher levels of SOD causes a return to the non-malignant phenotype.[51, 65] Nowadays, it was found that manganese-dependent superoxide dismutase (MnSOD) or SOD2 overexpression appears to enhance invasiveness and migration of malignant cells.[38,39] Moreover, several studies have reported that SOD2 is upregulated during tumor progression in prostate, colon and lung.[47] On the other hand, other studies report that SOD2 overexpression can suppress tumor incidence and proliferation in other cancers such as in breast and skin cancers. Innumerous polymorphic variants of these genes confer different expression and active proprieties on antioxidant cellular defense.[38, 39, 53-56] In colon cancer mutations, adenomatous polyposis, could begin a neoplasia process even when SOD2 levels are often low. The SODs activities are often lowered during early cancer development. However, *SOD2* mRNA and protein levels increase during early and intermediate stages in lung and colorectal cancers.[42, 47, 67, 69, 76, 77]

- ***SOD2* gene**

The role of SOD2 is still not exactly understood. SOD2, also named as MnSOD, regulates cellular redox homeostasis that is known to regulate proliferative and quiescent growth states. SOD2 expression is induced by a wide variety of factors such as hyperoxia, irradiation, cytokines (IL-1, TNF-α), oxidized LDL and the cellular redox state. Disturbances in the functioning of SOD isoforms lead to numerous pathological changes in the human organism, including tumor disease. Therefore, *MnSOD* and ROS rates are believed to be critical regulators.[41, 43, 50, 65-67, 76]

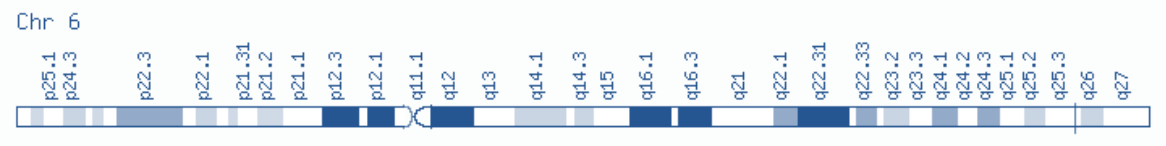


Figure 11: Genomic view of *MnSOD* gene. Adapted from: http://cdn.genecards.org/images/v4/genomic-location/SOD2-gene.png

The *SOD2* gene located in chromosome 6q25, it's the most well-known (Figure 11).[27, 43, 64, 76] The Val16Ala (47 T>C; rs4880) polymorphism has a single peptide mutation, a substitution of a valine acid for an alanine amino acid on its 16 codon. This mutation leads to a conformational change in the helical structure of the protein.[23,31] It is predicted that the Ala variant encodes proteins with a higher MnSOD activity than the Val variant, suggesting that Ala/Ala homozygous subjects may have higher SOD2 activity. Therefore, the defense response to oxidative stress will be altered as well.[23, 27, 31, 64, 76] It is hypothesized that the higher activity variant (Ala) suppresses carcinogenesis; however, this overproduction of SOD2 increases the levels of H_2O_2, as well. Epidemiologic studies associated the Ala variant with increased risk of carcinogenesis, particularly among people with lower intakes of exogenous antioxidants on their diet.[43, 76, 78]

There are several different views on how MnSOD expression can contribute to cancer development. Several authors reported that a loss of MnSOD activity results in aberrant proliferation, and was also related with poor 5-year overall survival.[31, 41, 65, 67, 68] One explanation for such discrepancies is the different clinical stages of the tumors studied.[66] CRC have been characterized

immunohistochemically by a decreased of MnSOD and total SOD activity when compared with adjacent normal mucosa, suggesting that MnSOD acts a tumor suppressor.[50] There are authors that showed a decreased or unchanged expression and activity of SOD isoenzymes.[43, 65, 74] While others observed increased expression and activity of SOD isoenzymes in various types of tumors, implying MnSOD in tumors progression, aggressiveness and metastatic potential.[66, 67, 72] MnSOD overexpression has shown to slow down cancer cell growth, but it also has a metastasis-promoting activity by the upregulation of matrix-degrading metalloproteases and blocking apoptosis.[44, 68, 78]

- ***SOD3* gene**

Extracellular superoxide dismutase (EC-SOD; SOD3) has Cu and Zn in the catalytic center and a heparin binding domain (HBD). SOD3 is highly expressed in plasma, blood vessels, heart, lungs, kidney, placenta and extracellular fluids. This enzyme is produced by resting macrophages and it's associated with the cell surface through extracellular matrix (ECM)-binding region.[76] SOD3 is a protective molecule which catalyzes the conversion of superoxide anions into hydrogen peroxide and oxygen, thus protecting from oxidative fragmentation of matrix components. Inherited change in SOD3 expression or function could affect organ matrix homeostasis and influence its normal function.[23, 31, 73, 76, 79, 80] SOD3 binds matrix components and inhibits their fragmentation in response to oxidative stress. Their fragmentation stimulate inflammatory cell migration, so SOD3 could play a central role in tissue defenses against oxidative stress.[23,79] It was observed that non-neoplastic tissue has more SOD3 expression than neoplastic tissue which supports the previously predicted role of SOD3 in tumorigenesis. Thus, dysregulation of extracellular oxidant-moderating proteins, such as SOD3, is significant in cancer. However, there is not much knowledge about EC-SOD in human tumors so further studies are needed in order to characterize its potential role.[81, 82, 83]

The most studied single nucleotide polymorphism (SNP) within *SOD3* is Arg231Gly (rs1799895; R213G) on chromosome 4 (Figure 12). About 2-3% of the population carries this polymorphism. The substitution of arginine to glycine in its

heparin binding domain at amino acid 213 (R213G) was first identified in patients with heart failure.[31, 73, 81, 82] This variant is known to reduce the binding capacity of SOD3 and may thus have an impact on the cellular distribution.[68, 76] Transcript levels of SOD3 significantly differed between tumor and non-neoplastic tissues. A low expression level of SOD3 induces primary cell proliferation and immortalization, whereas, high expression levels induce growth arrest, senescence, and apoptosis though signaling pathway activation (*p53* and *p21*).[84, 85] Despite the enzymatic activity of SOD3 should not be affect by this mutation, it was observed in lung adenocarcinoma that *SOD3* mRNA and protein expression were significant decreased.[75, 85]

Figure 12: Genomic view of *SOD3* gene.
Adapted from: http://cdn.genecards.org/images/v4/genomic-location/SOD3-gene.png

While *SOD2* was upregulated in the tumor tissue, this *SOD3* polymorphism leads to a higher concentration of SOD3 in plasma and lower in tissues, once it's anchoring/binding to the extracellular matrix is compromised. Thus SOD3 has strong downregulation in tumor tissue samples.[31, 78, 82, 86] Evidence of downregulation in SOD3 expression in lung and oral tumors, previously reported, have suggested that SO3 can have potential effects on extracellular regulation of multiple factors that regulate angiogenesis and invasion, increasing relative risk for this disease.[73, 77-80, 82-84, 87] It was reported that SOD3 overexpression, delays the onset of increased breathing frequency and significantly reduces breathing rates in wild-types mice exposed to a source of oxidative stress (ionizing radiation).[78] SOD3 overexpression also inhibits the invasive capacity of human prostate cancer cells.[82 78] Therefore, arginine 213 is critical for maintaining proper organ function through moderating the normal innate immune response, suggesting the potential to suppress aggressive tumor behavior.[78, 80, 82, 86]

1.5.2. Detoxify regulators: Glutathione S-transferases

Glutathione S-transferases (GSTs) super family is a family of Phase II detoxification enzymes that are involved in the detoxification of xenobiotic compounds. They catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Figure 13) formed during oxidative stress including those result from lipid peroxidation. [88-103]

Figure 13: Graphic representation of GSTs detoxify role.
Adapted from: Gorrini C. Harris IS. Mak TW. *Modulation of oxidative stress as an anticancer strategy.* Nature Reviews Drug Discovery 2013;12: 931–947

This process usually inactivates the electrophiles and facilitates their excretion into urine or bile.[88, 89] On the other hand, this detoxification ability plays an important role in cellular protection from environmental and oxidative stress, yet is also implicated in cellular resistance to drugs acquired by an accelerated detoxification of drug substrates.[90] The GSTs are found on liver and in the epithelium of the human gastrointestinal tract, thus providing a protective role for the cells in the gut.[88, 104, 105] GST activities may be reduced in colonic mucosa affected by chronic inflammatory conditions.[105] GSTs have also been shown to play critical roles in kinase signaling. However, this pathway is not fully understood. The lack of function of these enzymes has been correlated with a higher risk of cancer. Studies have linked aberrant expression of GST isozymes with the development and expression of resistance to a variety of chemicals. Therefore, differences in the human GST isozyme expression patterns influence cancer susceptibility, prognosis and treatment.[38, 53, 54, 88, 91-96, 98, 99, 100, 102, 106-108]

Glutathione S-transferases are high polymorphic among ethnicities. Human GSTs are divided into three main families with have similar ability to catalyze the conjugation of GSH: cytosolic, mitochondrial and membrane-bound microsomal. Based on identity sharing greater than 60% within a class and focuses mainly on the highly conserved N-terminal, the mammalian cytosolic family of GSTs is further

divided into seven classes*: alpha, mu, omega, pi, sigma, theta* and *zeta*.[90, 94, 96-98, 101, 104, 108-110]

Recently, was described plus one class – *lambda* (K).[103] From these classes above, alpha, mu, theta and pi have been identified in the human colon; however, the pi class predominates.[105, 110, 111] SNPs in those isoforms cause a steric change at the substrate-binding site of the enzyme that changes its catalytic activity which seems to denote impaired ability to detoxify carcinogens, conferring an increased cancer risk.[90, 110, 112]

- ***GSTP* gene**

The *GST Pi* class is encoded by a single gene located on chromosome 11 (Figure 14). Four functionally different polymorphisms have been identified (GSTP1*A–D). For *GSTP1*, two genetic polymorphisms are known for this gene: in exon 5, a substitution A>G at codon 105 of GSTP*B gene (Ile105Val; rs1695), turns amino acid isoleucine to valine; in exon 6, transition of C>T at codon 114 turns in a substitution of amino acid alanine to valine (Ala114Val; rs1138272).[27, 90, 94-97, 106-108,110, 111] These SNPs cause a steric change at the substrate-binding site of the enzyme that changes its catalytic activity without affecting the binding affinity to glutathione.[90, 110]

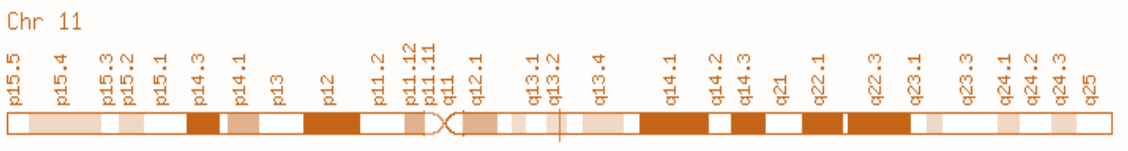


Figure 14: Genomic view of *GSTP* gene.
Adapted from: http://cdn.genecards.org/images/v4/genomic-location/GSTM1-gene.png

The *GSTP1* genotype has been associated with differences in chemotherapeutic response and cancer susceptibility and is overexpressed in a wide variety of tumors including ovarian, bladder, testicular, lung, breast, colon, pancreas, larynx and lymphoma.[94, 95, 97, 105, 110] GST expression and/or activity of

specific isoforms are lost in some individuals with allelic variation and are proposed to occur during pathogenesis of the disease. Lack of GSTP1 expression results on enzyme activity reduction, followed by an increase of cellular oxidative stress which allows the human colon cancer cell survival and proliferation.[94, 102, 110] The Ile105Val results in a catalytic activity reduction and thus a diminished detoxification capacity in individuals V105 when compared with I105.[97,112] It was observed a slightly better survival in GSTP1 Ile/Ile patients compared with patients with the Ile/Val or Val/Val genotypes.[98,112] However, the association between the *Glutathione S transferase-P1 (GSTP1) Ile105Val* polymorphism and colorectal cancer (CRC) susceptibility are still inconsistent between studies.[102, 106, 110] *GSTP1 Val114* polymorphism apparently contributes to esophageal and colorectal cancer susceptibility.[102, 110]

- ***GSTT* gene**

The Theta class of GSTs consists of two different subfamilies: GSTT1 and GSTT2. Genes encoding both proteins are localized on chromosome 22 (Figure 15). Polymorphisms exist within both genes. *GSTT1* has a functional and a non-functional allele. Homozygosity for the nonfunctional allele of *GSTT1* is the null phenotype and exhibits an absence of catalytic activity. Individuals homozygous for this deletion are thought to be at increased risk for malignancies (such as cancer, cardiovascular and respiratory diseases) as a consequence of a decreased capacity to detoxify possible carcinogens.[95, 94, 99, 101-104, 113, 114] It was demonstrated that 20% of Caucasians carries this genotype.[114] The *GSTT1* polymorphism was significant in breast cancer and for CRC risk in Caucasians.[95, 101, 115] However, this association is not consistent between all studies reported.

Figure 15: Genomic view of *GSTT* gene.
Adapted from: http://www.ncbi.nlm.nih.gov/gene/2952

- ***GSTM* gene**

Five GSTM isoforms belonging to the mu class (GSTM1- 5) have been described. A gene cluster located on chromosome 1 encodes for *GSTM1-5*, four different alleles allowing for several M1 class polymorphisms (Figure 16). The presence of the *GSTM1* allele has been associated with a decreased risk of bladder cancer.[91, 94, 104] Loss of GSTM enzyme function is described to a homozygous deletion of this gene resulting in the GSTM1 null allele. It was showed that 20-50% of Caucasians carries *GSTM1 null* genotype.[114, 115] The *GSTM null* phenotypes are associated with an increased risk of the lung, head, colon and bladder cancer and were also been associated with response rates to some chemotherapy. Data have suggested that *GSTM1* polymorphism is associated with an increased risk of CRC, especially in the Caucasian population.[88, 89, 94-96, 99, 103-105,108, 114]

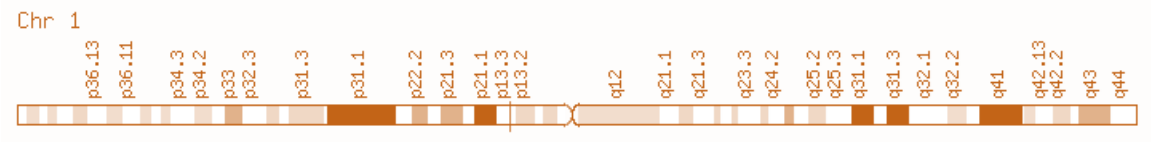


Figure 16: Genomic view of *GSTM* gene.
Adapted from: http://cdn.genecards.org/images/v4/genomic-location/GSTM1-gene.png

1.6. Antioxidant and Detoxify regulators in colorectal cancer

The gene variants can affect the risk of sporadic colorectal cancer associated with various environmental and dietary factors. Diet and social behavior are a fundamental risk factor for development of CRC, and its influence appears to be stronger during post-initiation phases of carcinogenesis. Accordingly, antioxidant and detoxify molecular mechanisms should be considered among potential risk modulators for this neoplasia. Several gene polymorphisms involved in regulation detox defense. However, there still inconsistencies on CRC ethology.

Both antioxidant and detoxify genes are crucial to redox hemostasis. Imbalance on these defense mechanisms leads to ROS accumulation and predispose DNA cell to damage. This damage can change protein activity and function. ROS accumulation on colorectal environment predisposes to risk of CRC. The balance between antioxidants and ROS is lost. Therefore, colonic and rectal cells will be prone to DNA damage. This damage induces changes on enzymatic function and activity. The continuous exposure of colonic and rectal cells to ROS damage will allow to neoplasic development (Figure 17). The *MNSOD, SOD3, GSTP1, GSTT1* and *GSTM1* genes have an important role in antioxidant and detoxify metabolisms management. Numerous studies reported that their polymorphic variants have an huge impact on their functions. However, some studies about association and influence on CRC didn't agree with majority.

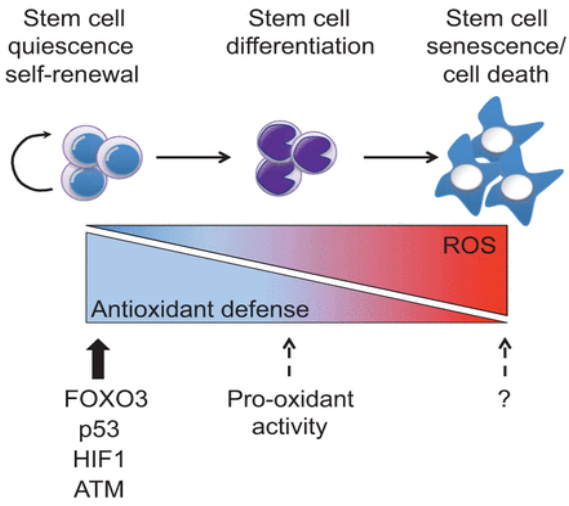


Figure 17: Colonic and rectal cells neoplastic development induced by continuous ROS exposure. Adapted from: Bigarella CL. Liang R. Ghaffari S.. Stem cells and the impact of ROS signaling. Development. 2014; 141(22): 4206–4218.

The knowledge about detox genes polymorphisms involved on CRC genesis and development will be essential for new approaches on cancer research. However, Sporadic Colorectal Adenocarcinoma (SCA) origin hasn't been studied until now. Therefore, we wanted verify if *MNSOD, SOD3, GSTP1, GSTT1* and *GSTM1* gene polymorphisms influence the risk of SCA occurrence. Moreover, we want to know if those polymorphisms are associated to gender and colonic and rectal localization among SCA subjects.

II. Material and methods

2.1. Material

A global of 68 samples of SCA (mean age of 67 +/- 18 years; 75% men and 25% women) was used in the research of the defined polymorphisms (Table 1). A total of 31 colon and 37 rectal biopsies suffered the typical process of fixation in formaldehyde and inclusion in paraffin and were selected from the archives of Anatomical Pathology Institute of Faculty de Medicine of University of Coimbra (Figure 18). Samples were collected between 2009 and 2011, tumor diagnosis and differentiation grade of the tumor were established by histological evaluation of tumor fragments using criteria according to World Health Organization (Table 2).

A total of 100 healthy subjects biopsies (with normal colic mucosa) were used as control group (mean age of 73 +/- 9 years; 79% men and 21% women) (Table 1). This study was supported and approved by local ethics committee (CIMAGO - Faculty of Medicine of the University of Coimbra, Coimbra, Portugal).

					Tumor location				
		Distribution	Age		Colon		Rectum		**Total**
		%	Mean	Stand. Dev.	n	%	n	%	
	Gender								
Patients	Male	75	67	18	25	81	26	70	51
	Female	25			6	19	11	30	17
	Total	100			31	100	37	100	68
Controls	Male	79	73	9					
	Female	21							
	Total	200							

Table 1: Clinical data of SCA and control samples.

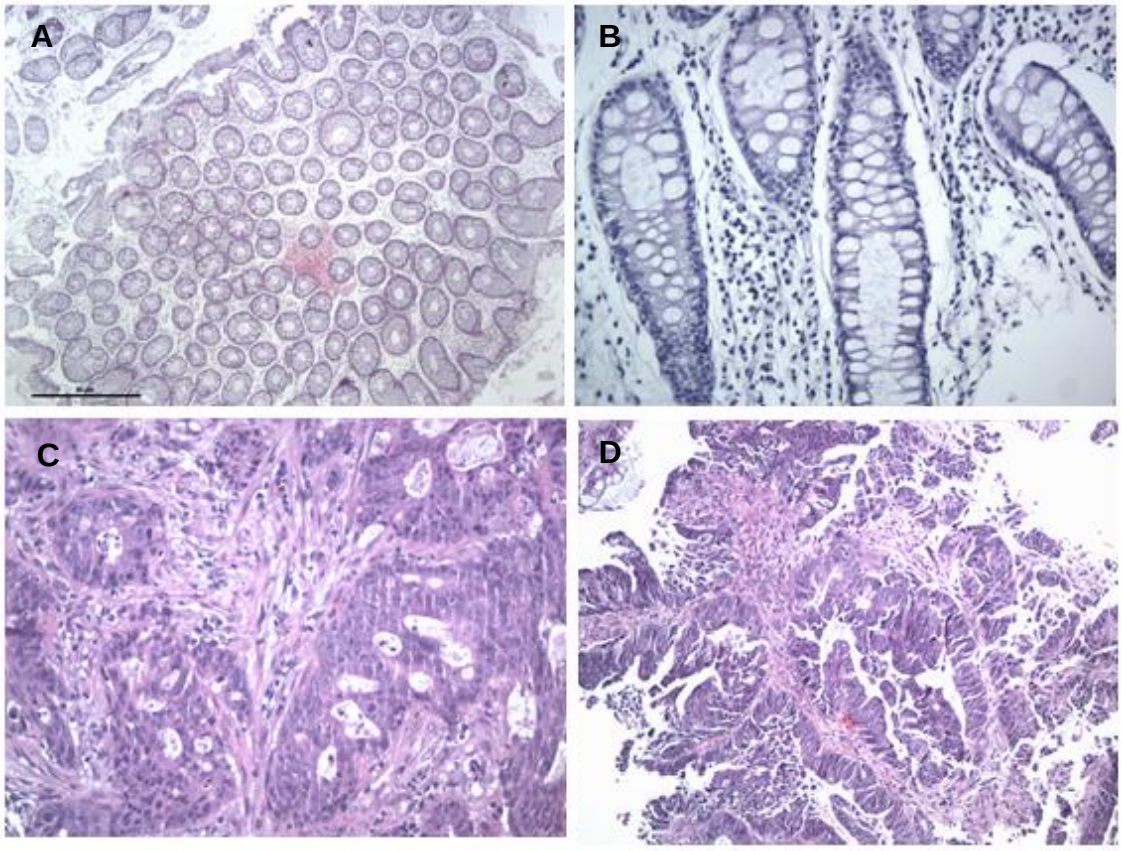


Figure 18: (A) and (B) Histologic picture of normal colonic mucosa: 40x HE, 200x HE; (C) and (D) – Histological picture of well differentiated sporadic adenocarcinoma. 200x HE (hematoxylin-eosine); 100x HE. Images from IAP-FMUC.

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

Table 2: Tumor grade according to World Health Organization.

2.2. Methods

2.2.1. DNA extraction

DNA genomic isolation from biopsies were made according to the extraction protocol from *NZY Tissue gDNA Isolation Kit* (NZYTech, Lisbon, Portugal), after microdissection of the normal colic tissue (5 to 10 dissections of 10µm of thickness to each sample). Samples were prepared by adding 1 ml of xylene to each tube to paraffin removal. After centrifugation at 11,000x*g* for 3 min, supernatant was discarded and samples were washed with 1ml of ethanol (96%-100%), repeating the centrifugation step. Lysis of the cell wall by Proteinase K and NZY was then performed overnight and purification of DNA obtained through columns and according to the procedure of the referred kit. The DNA was further stored at –20ºC.

2.2.2. 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 280 nm, while at the 260 nm wavelength there is detection of nucleotides only. At a wavelength of 230 nm, the presence of contaminants is assessed.

2.2.3. 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. *MNSOD* T175C*, SOD3* R213G, *GSTP1* A105G, *GSTP1* C114T, *GSTT1* del, *GSTM1* del mutations detection 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	95º C	25 Sec	5
Annealing	70º C	45 Sec	
Extension	72º C	30 Sec	
Denature	95º C	25 Sec	21
Annealing	65º C	45 Sec	
Extension	72º C	30 Sec	
Denature	95º C	25 Sec	4
Annealing	55º C	1 Min	
Extension	72º C	2 Min	
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.

2.2.4. Electrophoresis in agarose gel

PCR reactions, after amplification, were submitted to electrophoresis by 2% agarose gel in order to identify the amplified products. Agarose *Routine Grade* (NZYTech, Lisbon, Portugal) was dissolved in 1x TAE (Tris-acetate-EDTA) (NZYTech, Lisbon, Portugal) and distillated water and agitated for 15 seconds. Then the solution was transferred to the microwaves to complete dissolution of agarose (approximately 2 min at 900w). Afterwards, the solution was cool down and $1x10^{-5}$ SYBR Safe (Molecular Probes, Oregon – USA) a dye that allows the visualization of DNA under the UV light incidence, were added and the solution was agitated for 15 seconds for homogenization. Solution was after putted into a cradle 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). PCR samples were 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, PCR products were visualized under UV light by a transilluminator (UVi Tech, Cambridge, United Kingdom). Results were further registered by a digital camera (NIKON DMX1200F).

2.2.5. Statistical analysis

2.2.5.1. Analysis of purity and concentration of DNA

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

2.2.5.2. Frequencies of allele and genotype 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. *MnSOD* T175C, *SOD3* R213G, *GSTP1* A105G, *GSTP1* C114T, *GSTT1* del*, GSTM1* del frequencies were compared between different groups (SCA versus control and among tumor 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.

III. Results

3.1. 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 1. The distribution of SCA subjects by gender shows the predominance of the disease in male subjects comparing with female individuals (75% versus 25%) (Table 1). In terms of tumors localization, it was observed a small difference between gender distribution among colon and rectum groups, however, no significant differences were found (Table 4). Moreover, 46% of the SCA biopsies were located in colon and 54% of the SCA biopsies were located in rectum (Table 4). However, there were no significant differences between mean age in both groups (patient samples: mean age of 67 +/- 18 years; controls: mean age of 73 +/- 9 years) (Table 1).

	Colon		**Rectum**				
	n	%	N	%	*p*	OR	RR
Male	25	81	26	70			
Female	6	19	11	30		NS	
Total	31	46	37	54			

Table 4: Gender distribution among colon and rectum groups.

3.2. Analysis of purity and concentration of DNA

Although the concentration of DNA is not uniform (+/-29.3μg/ml) and lower than the standard value (100 μg/ml) (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 and 0.4-0.6 to O.D.230nm/O.D.260nm, some DNAs show high quantities of contaminants and proteins with a confidence interval being above certain acceptable limits.

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). In general, PCR-SSP didn't have 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	29.3	0.09	0.15
Variation Coefficient	69%	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.

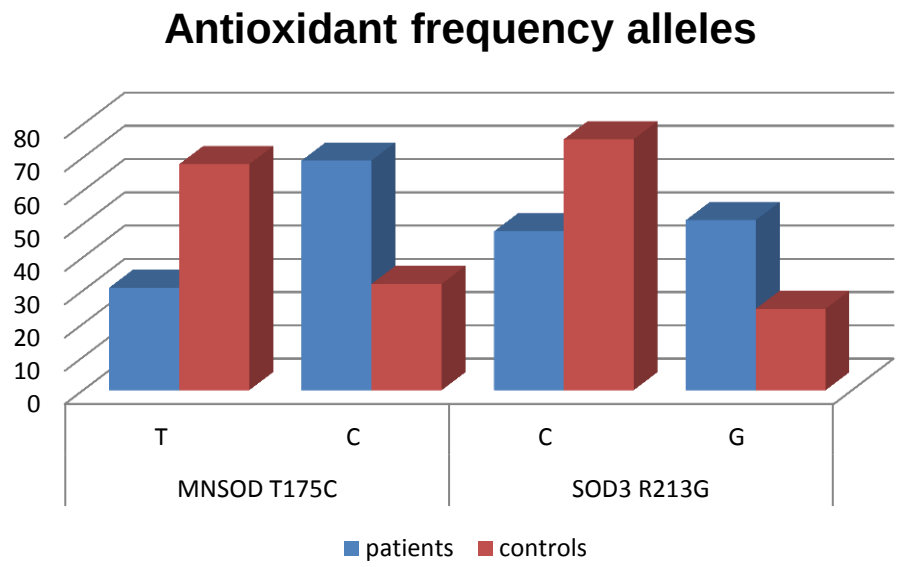
3.3. Molecular analysis of antioxidant and detoxify genes

3.3.1. Polymorphisms in SCA and controls

When compared SCA with controls, we notice that *MNSOD* 175C (p<0.0001; 69% vs 32%; OR:4.76; CI: 2.97 to 7.61), *SOD3* 213G (p<0.0001; 52% vs 25%; OR:3.37; CI: 2.11 to 5.36), *GSTP1* 105G (p<0.0001; 59% vs 30%; OR:3.33; CI: 2.11 to 5.26) and *GSTP1* 114T (p<0.0001; 53% vs 12%; OR:8.66; CI: 5.00 to 15.00) mutant alleles were more frequent among SCA subjects (Table 6-7; Graphic 1-2).

	Patients and controls						
Alleles	Patients		Controls		*p*	OR	RR
	n	%	n	%			
MNSOD T175 C (rs4880)							
T	42	31	136	68	<0.0001	0.21 (0.13-0.34)	0.40 (0.30-0.53)
C	94	69	64	32		4.76 (2.97-7.61)	2.52 (1.90-3.38)
Total	136	100	200	100			
SOD3 R213G (rs1799895)							
C	65	48	151	75	<0.0001	0.30 (0.20-0.47)	0.51 (0.40-0.65)
G	71	52	49	25		3.37 (2.11-5.36)	1.97 (1.53-2.53)
Total	136	100	200	100			

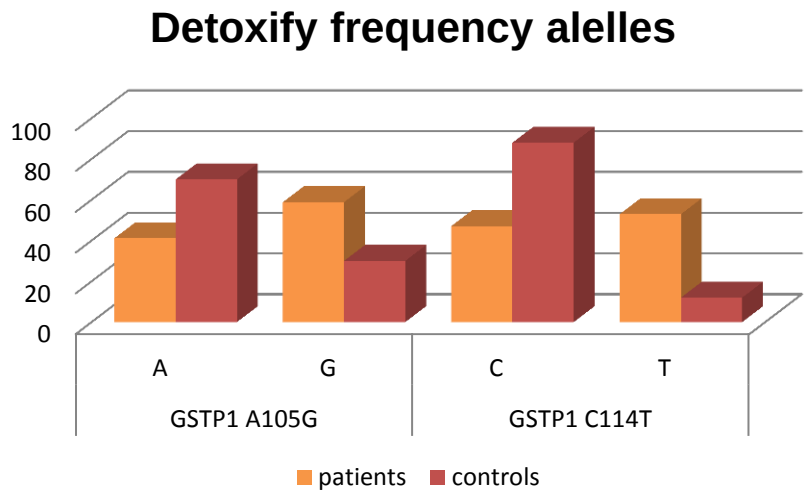
Table 6: Allelic frequency of antioxidant genes in SCA and control group.



Graphic 1: Allelic frequency of antioxidant genes in SCA and control group (p<0.0001).

Alleles	Patients and controls						
	Patients		Controls		*p*	OR	RR
	n	%	n	%			
GSTP1 A105G (rs1695)							
A	56	41	140	70	<0.0001	0.30 (0.19-0.47)	0.50 (0.38-0.65)
G	80	59	60	30		3.33 (2.11-5.26)	2 (1.54-2.60)
Total	136	100	200	100			
GSTP1 C114T (rs1138272)							
C	64	47	177	88	<0.0001	0.12 (0.07-0.20)	0,35 (0.28-0.45)
T	72	53	23	12		8.66 (5.00-15.00)	2.85 (2.25-3.62)
Total	136	100	200	100			

Table 7. Allelic frequency of detoxify genes in SCA and control group.

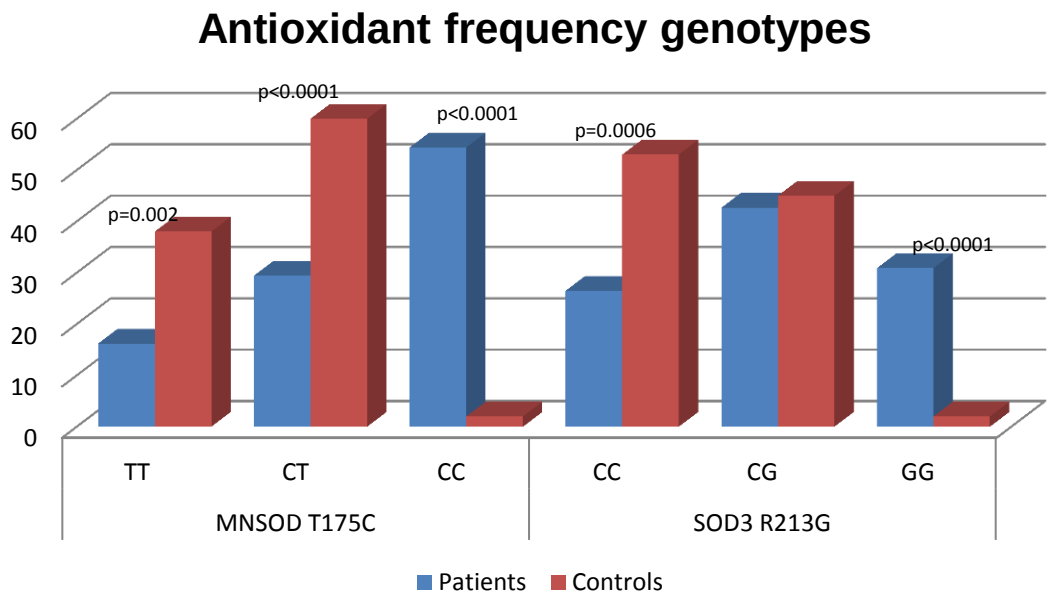


Graphic 2: Allelic frequency of antioxidant genes in SCA and control group (p<0.0001).

We also found a higher prevalence of *MNSOD* 175CC (55% vs 2%; p<0.0001; OR: 58.5; CI 13.3 to 256.7), *SOD3* 213GG (31% vs 2%; p<0.0001; OR: 21.89; CI 4.93 to 97.29), *GSTP1* 105GG (46% vs 12%; p<0.0001; OR: 6.14; CI 2.85 to 13.26), *GSTP1* 114TT (38% vs 0%; p<0.0001; OR: Infinity) and *GSTT1* null (75% vs 28%; p<0.0001; OR: 7.71; CI 3.83 to 15.56) mutated genotypes between SCA patients. *GSTM1* del mutated genotype was not statistical significance between SCA patients and control group (Table 8-9; Graphic 3-4).

Patients and controls							
Genotypes	Patients		Controls		*p* value	OR	RR
	n	%	n	%			
MNSOD T175 C (rs4880)							
TT	11	16	38	38	0.002	0.32 (0.15-0.67)	0.47 (0.27-0.82)
TC	20	29	60	60	<0.0001	0.30 (0.20-0.47)	0.51 (0.40-0.65)
CC	37	55	2	2		59 (13.3-256.7)	3.95 (2.88-5.41)
Total	68	100	100	100			
SOD3 R213G (rs1799895)							
CC	18	26	53	53	0.0006	0.32 (0.16-0.62)	0.49 (0.32-0.77)
CG	29	42	45	45		NS	
GG	21	32	2	2	<0.0001	21.9 (4.93-97.29)	2.82 (2.16-3.68)
Total	68	100	100	100			

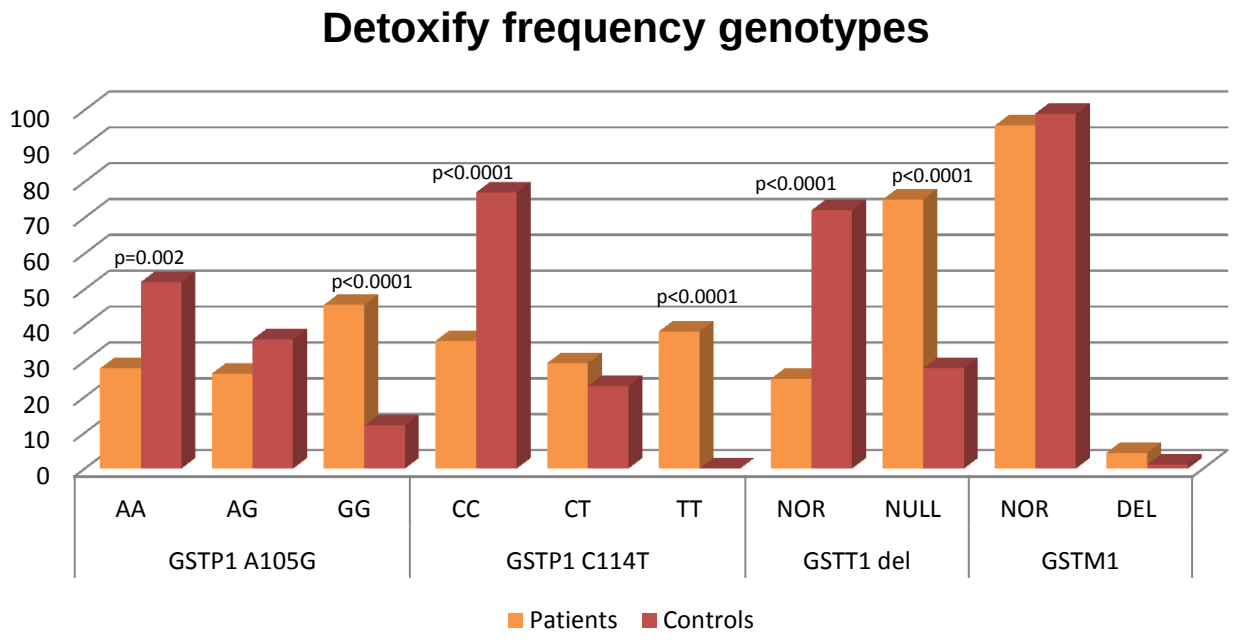
Table 8: Genotype frequency of antioxidant genes in SCA and control group.



Graphic 3: Genotype frequency of antioxidant genes in SCA and control group.

Patients and controls							
Genotypes	Patients		Controls		*p* value	OR	RR
	n	%	n	%			
GSTP1 A105G (rs1695)							
AA	19	28	52	52	0.002	0.36 (0.19-0.69)	0.53 (0.34-0.82)
AG	18	26	36	36		NS	
GG	31	46	12	12	<0,0001	6.14 (2.85-13.26)	2.44 (1.75-3.38)
Total	68	100	100	100			
GSTP1 C114T (rs1138272)							
CC	22	36	77	77	<0,0001	0.14 (0.07-0.29)	0.33 (0.23-0.50)
CT	20	30	23	23		NS	
TT	26	38	0	0	<0,0001	Infinity	3.4 (2.62-4.36)
Total	68	100	100	100			
GSTT1del							
NOR	17	25	72	72	<0.0001	0.13 (0.06-0.26)	0.30 (0.19-0.47)
NULL	51	75	28	28		7.71 (3.83-15.56)	3.38 (2.14-5.34)
Total	68	100	100	100			
GSTM1del							
NOR	65	96	99	99		NS	
NULL	3	4	1	1			
Total	68	100	100	100			

Table 9: Genotype frequency of detoxify genes in SCA and control group.



Graphic 4: Genotype frequency of detoxify genes in SCA and control group.

3.4. Patient's polymorphisms stratified by:

3.4.1. Gender

The distribution of SCA subjects by gender shows no significant differences between men and women (Tables 10-13).

Alleles	Gender				p	OR	RR
	Male		Female				
	n	%	n	%			
MNSOD T175 C (rs4880)							
T	32	31	10	29		NS	
C	70	69	24	71			
Total	102	100	34	100			
SOD3 R213G (rs1799895)							
C	45	44	20	59		NS	
G	57	56	14	41			
Total	102	100	34	100			

Table 10: Allelic frequency of antioxidant genes by gender.

Alleles	Gender				p	OR	RR
	Male		Female				
	n	%	n	%			
GSTP1 A105G (rs1695)							
A	38	37	18	53		NS	
G	64	63	16	47			
Total	102	100	34	100			
GSTP1 C114T (rs1138272)							
C	48	47	16	47		NS	
T	54	53	18	53			
Total	102	100	34	100			

Table 11: Allelic frequency of detoxify genes by gender.

Genotypes	Gender						
	Male		Female		p	OR	RR
	n	%	n	%			
MNSOD T175 C (rs4880)							
TT	9	18	2	12			
CT	14	27	6	35		NS	
CC	28	55	9	53			
Total	51	100	17	100			
SOD3 R213G (rs1799895)							
CC	12	24	6	35			
CG	21	41	8	47		NS	
GG	18	35	3	18			
Total	51	100	17	100			

Table 12: Genotype frequency of antioxidant genes by gender.

Genotypes	Gender						
	Male		Female		p	OR	RR
	n	%	n	%			
GSTP1 A105G (rs1695)							
AA	12	24	7	41			
AG	14	27	4	24		NS	
GG	25	49	6	35			
Total	51	100	17	100			
GSTP1 C114T (rs1138272)							
CC	16	31	6	35			
CT	16	31	4	24		NS	
TT	19	38	7	41			
Total	51	100	17	100			
GSTT1del							
NOR	12	24	5	29			
NULL	39	76	12	71		NS	
Total	51	100	17	100			
GSTM1del							
NOR	46	90	17	100			
NULL	5	10	0	0		NS	
Total	51	100	17	100			
GSTM del							
NOR	7	14	2	12			
NULL	44	86	15	88		NS	
Total	51	100	17	100			

Table 13: Genotype frequency of detoxify genes by gender.

3.4.2. Tumor localization

Allele frequencies from polymorphisms of *MNSOD, SOD3, GSTP, GSTT and GSTM* genes in SCA subjects and controls are presented in Tables 6 and 7. The majority of genes did not present a significant difference between groups. Only *GSTP1* alleles had significant difference between colon and rectum samples (p=0.03), being the mutated allele *GSTP1 114T* more prevalent among colon samples (63% vs 45%; p=0.03; OR: 2.11; CI 1.06 to 4.20) (Table 14).

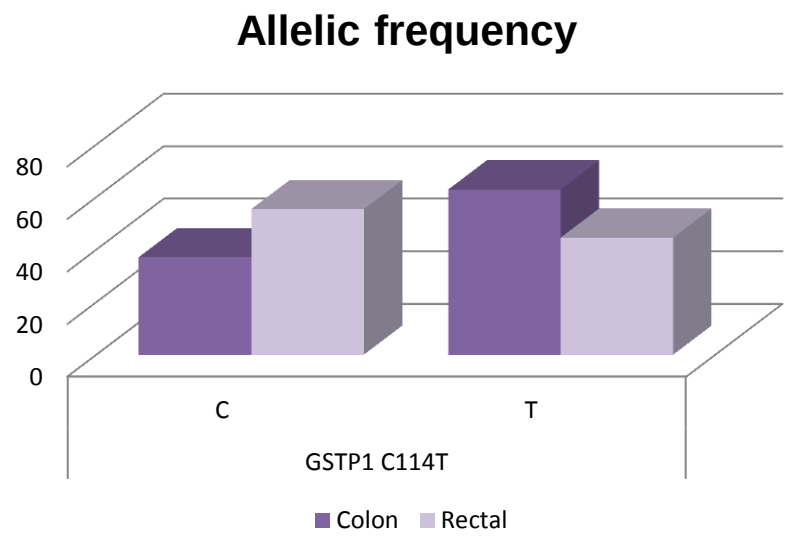
Allele	Tumor localization						
	Colon		Rectal		*p*	OR	RR
	n	%	n	%			
MNSOD T175 C (rs4880)							
T	21	34	21	28		NS	
C	41	66	53	72			
TOTAL	62	100	74	100			
SOD3 R213G (rs1799895)							
C	28	45	37	50		NS	
G	34	55	37	50			
TOTAL	62	100	74	100			
GSTP1 A105G (rs1695)							
A	28	45	28	38		NS	
G	34	55	46	62			
TOTAL	62	100	74	100			
GSTP1 C114T (rs1138272)							
C	23	37	41	55	0.03	0.48 (0.24-0.95)	0.72 (0.52-0.98)
T	39	63	33	45		2.11 (1.06-4.20)	1.40 (1.02-1.91)
TOTAL	62	100	74	100			

Table 14: Colon-rectal allele frequency in SCA patients

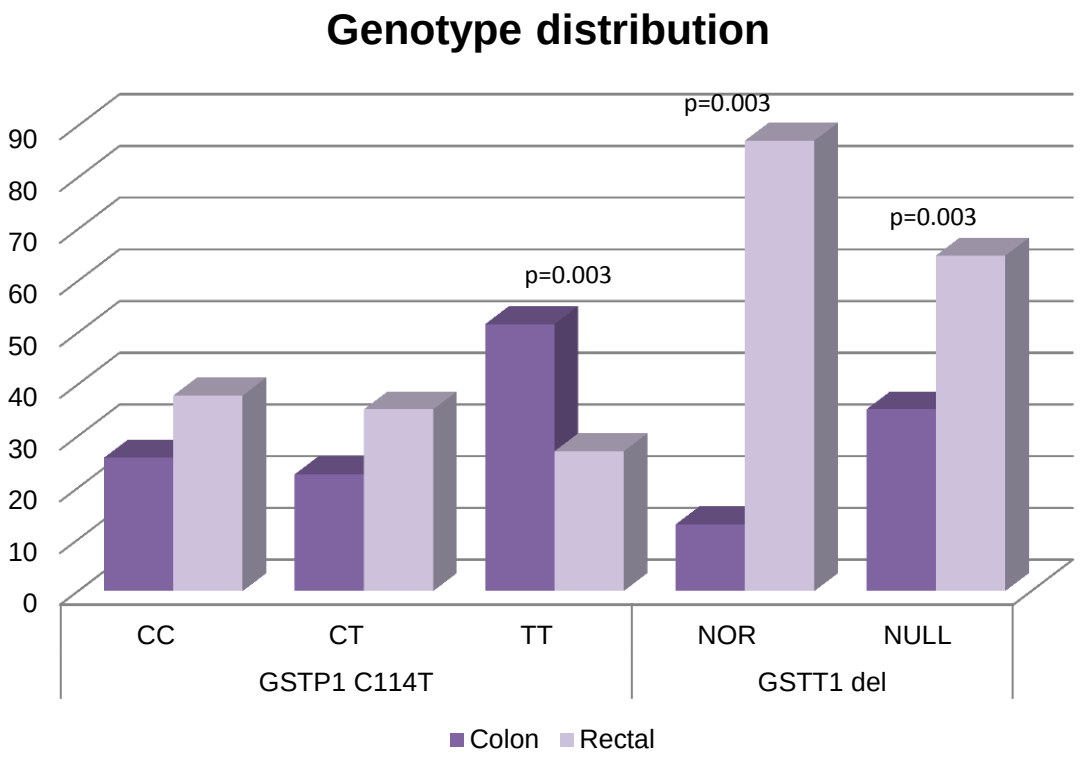
In the same way, we found *GSTP1* 114TT mutated genotype associated with colon samples (52% vs 27%; p=0.003; OR: 2.88; CI: 1.41 to 5.89). *GSTT1* null genotype were also prevalent among colon samples (87% vs 65%; p=0.003; OR: 3.66; CI 1.51 to 8.84) while the normal genotype were associated with rectum samples (Table 15; Graphic 5-6).

Genotypes	Tumor localization						
	Colon		Rectal		*p*	OR	RR
	n	%	n	%			
MNSOD T175 C (rs4880)							
TT	10	16	12	16			
TC	22	36	18	24		NS	
CC	30	48	44	60			
TOTAL	62	100	74	100			
SOD3 R213G (rs1799895)							
CC	16	26	20	27			
CG	24	39	34	46		NS	
GG	22	35	20	27			
TOTAL	62	100	74	100			
GSTP1 A105G (rs1695)							
AA	18	29	20	27			
AG	20	32	16	22		NS	
GG	24	39	38	51			
TOTAL	62	100	74	100			
GSTP1 C114T (rs1138272)							
CC	16	26	28	38		NS	
CT	14	22	26	35			
TT	32	52	20	27	0.003	2.88 (1.41-5.89)	1.72 (1.20-2.47)
TOTAL	62	100	74	100			
GSTT1 del							
NOR	8	13	26	35	0.003	0.27 (0.11-0.66)	0.62 (0.47-0.81)
NULL	54	87	48	65		3.66 (1.51-8.84)	1.63 (1.23-2.15)
TOTAL	62	100	74	100			
GSTM1 del							
NOR	32	52	40	54		NS	
NULL	30	48	34	46			
TOTAL	62	100	74	100			

Table 15: Colon-rectal genotype frequency in SCA patients.



Graphic 5: Colon-rectal allelic frequency of GSTP gene in SCA patients (p=0.03).



Graphic 6: Colon-rectal genotype frequency of GSTP gene in SCA patients.

IV. Discussion

SCA development is the result of a complex interaction of variables, including external factors such as exposure to environmental agents and dietary factors and internal factors. Sporadic colorectal cancer is influenced by the local colonic environment and the patient's genetic background. It has been established that genotypes with low penetrance polymorphisms are related with detox metabolism and thus with risk of CRC. However, it is unknown their role on sporadic colorectal adenocarcinoma. For this reason, the aim of this study was to determine whether these genetic polymorphisms influence the risk of SCA and their clinical/biological importance.

Between the genes with antioxidant and detoxifying activity, *superoxide dismutases (SOD)* and *glutathione-S-transferases (GST)* genes are highly polymorphic and often contain numerous mutations, increasing the risk of the cancer development. Therefore, the study of *MNSOD, SOD3, GSTP, GSTT* and *GSTM* genotypes, involved in detox metabolism, is very important to clarify oxidative stress involvement in SCA development. These genes encode enzymes that can have their activity changed due mutations, on their variant alleles. These mutations are substitutions or deletions that encode different amino acids which have their enzymatic function altered or lost. These changes consequently lead to the alteration in the risk of malignant diseases, whether conferring a certain protection or increasing the risk of development of the disease due the accumulation of ROS.

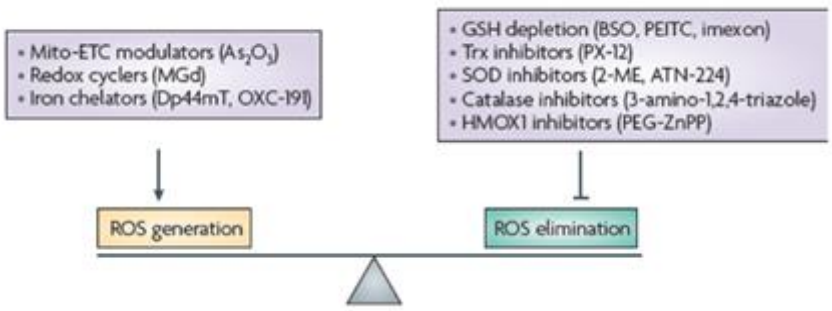


Figure 19 A

In this view, we supposed that some antioxidant and detoxify polymorphisms would be involved in oxidative stress regulation (Figure 19 A-B). They may predispose to disease and particularly on SCA. The strength of the biological impact will depend on heterozygosity or homozygosity of the variant allele.

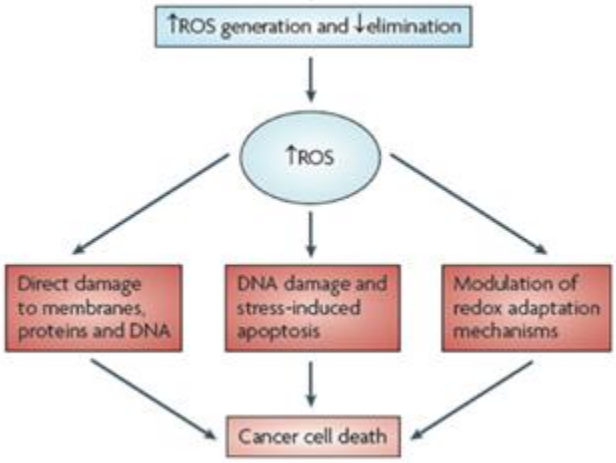


Figure 19 B

Figure 19 A-B: ROS accumulation disturbs oxidative regulation. For our turn, this imbalance leads to cancer development.
Adapted from: Graves DB. *The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology*. J Phys D Appl Phys. 2015; 45: 263001

4.1. SCA subjects and controls

4.1.1. Antioxidant genes

Superoxide dismutases seem to have an important role on oxidative metabolism balance. They are the first line of enzymes responsible for antioxidant defense. SODs catalyze O_2^- to H_2O_2 through reduction and re-oxidation of the metal on their active center. Loss of activity leads to ROS accumulation. From the three main isoforms, *SOD2* and *SOD3* have been associated with carcinoma.[23, 38, 39, 43, 53, 56, 64, 75] The innumerous polymorphic variants of these genes confer different expression and active proprieties on antioxidant cellular defense.[38, 39, 53-56] Our results suggest that both *SOD2* and *SOD3* influence SCA risk.

- ***SOD2* gene**

Several researches suggest that *MnSOD* genes influence CRC risk. This hypothesis is corroborated with our results. We observed a high correlation in mutant C alleles (69% vs 32%; p<0.0001; OR:4.76; RR 2.52) and CC genotypes 175CC (54% vs 2%; p<0.0001; OR: 58,5; CI 13,3 to 256,7) in SCA patients when compared with control group. This mutation encodes an Ala variant with higher activity than the Val variant, suggesting that Ala/Ala genotypes subjects may have higher SOD2 activity. MnSOD, found in mitochondria, regulates cellular redox homeostasis that is known to regulate proliferative and quiescent growth states. Few studies have also reported that SOD2 is upregulated during tumor progression in prostate, colon and lung.[76] SOD2 overexpression appears to enhance invasiveness and migration of malignant cells.[76] Therefore, this overproduction of SOD2 increases the levels of H_2O_2 increasing cancer risk. Other authors observed an increased expression and activity of SOD isoenzymes in various types of tumors, implying MnSOD in tumors progression, aggressiveness and metastatic potential.[66, 67, 72] However, there are still some differences between studies. The different clinical stages of the tumors studied could justify this inconsistences.[66]

Furthermore, we can also observe that T allele, with normal activity levels, seems to have a protective effect on SCA. While C allele, over activated protein, increases the SCA risk.

- ***SOD3* gene**

SOD3 is an extracellular enzyme highly expressed in plasma, blood vessels, heart, lungs, kidney, placenta and extracellular fluids. Like SOD2, it is a protective molecule that catalyzes the conversion of superoxide anions into hydrogen peroxide and oxygen. SOD3 binds to extracellular matrix protecting from oxidative fragmentation. It was observed that non-neoplastic tissue has more SOD3 expression than neoplastic tissue which supports the role of SOD3 in tumorigenesis. However, there is not much knowledge about EC-SOD in human tumors.[77, 81, 82] SOD3 variant is known to reduce the binding capacity of SOD3. So transcript levels of SOD3 should differ between tumor and non-neoplastic tissues.

According to this, we observed a prevalence of *SOD3* 213G mutated allele (p<0.0001; 52% vs 25%; OR: 3.37; RR: 1.97) and *SOD3* 213GG mutated genotype (31% vs 2%; p<0.0001; OR: 21.89; CI 4.93 to 97.29). We can see that C allele is dominant, thus G allele has significance only when found in homozygous. It is expected that anchoring/binding to the extracellular matrix is compromised. Thus, like we saw, *SOD3* is diminished in tumor tissue samples. Consequently, SOD3 would be free on plasma leaving matrix prone to fragmentation and DNA damage by ROS. On the other hand, this fragmentation drives to an immune response via inflammatory cell migration.[23, 31, 78, 79, 82, 86] We also observed that 2% of our group control had *SOD3* R213G polymorphism which is according with the 2-3% described on literature. Also, we can observe that C allele, with binding capacity to extracellular matrix, seems to have a protective effect on SCA. While G allele, without anchoring capacity, increases the SCA risk

4.1.2. Detoxify genes

Glutathione S-transferase (GST) is a super family of Phase II detoxification enzymes involved in the detoxification of xenobiotic compounds. They are responsible for glutathione (GSH) conjugation to a wide variety of endogenous and exogenous electrophilic compounds formed during oxidative stress, such as, from lipid peroxidation.[88-103] Electrophiles inactivation facilitates their excretion into urine or bile.[88, 89] GST activities may be reduced in colonic mucosa affected by chronic inflammatory conditions.[105] The lack of function of these enzymes has been correlated with a higher risk of cancer.

Glutathione S-transferases are high polymorphic among ethnicities. The mammalian cytosolic family of GSTs is dividing into seven classes. From those classes pi, theta and mu have been identified in the human colon; when, the pi class predominates.[105, 110, 111] SNPs in those isoforms change its enzyme catalytic activity which seems to denote impaired ability to detoxify carcinogens, conferring an increased cancer risk.[90, 110, 112]

- ***GSTP* gene**

The *GSTP1* genotype is overexpressed in a wide variety of tumors including ovarian, bladder, testicular, lung, breast, colon, pancreas, larynx and lymphoma.[94, 95, 97, 105, 110] We search for two genetic polymorphisms in *GSTP1*: Ile105Val and Ala114Val. GST expression and/or activity are lost in some individuals with those allelic variations. This change GSTP1 catalytic activity but no it's binding affinity to glutathione.[90, 110] A reduction of enzyme activity is followed by an increase of cellular oxidative stress which allows the human colon cancer cell survival and proliferation.[94, 102, 110] However, the association between the *Glutathione S transferase-P1 (GSTP1)* Ile105Val polymorphism and colorectal cancer (CRC) susceptibility are still inconsistent between studies.[102, 110, 113]

In our study, we notice that *GSTP1* 105G mutated allele (P<0.0001; 59% vs 30%; OR: 3.33; RR:2) and *GSTP1* 105GG mutated genotype (46% vs 12%; p<0.0001; OR: 6.14; CI 2,85 to 13,26) were frequent among SCA subjects. We can see that A allele is dominant, thus G allele has only significance when found in homozygous. *GSTP1* 105Val polymorphism has a catalytic activity reduction and thus a diminished detoxification capacity in individuals with G mutated allele when compared with wild-type.[97,112] Thus, we can presume that A allele, with normal activity levels, seems to have a protective effect on SCA. While G allele, with lower activity, increases the SCA risk.

In the same way, *GSTP1* 114T (p<0.0001; 53% vs 12%; OR: 8.66; RR 2.85) mutant allele and *GSTP1* 114TT (38% vs 0%; p<0.0001; OR: Infinity) mutated genotype were widespread in SCA subjects. Mutated T allele is associated with SCA risk and mutated TT genotype has a great association with this disease. In SCA we had 38% homozygous mutated polymorphisms whereas in our group control there was none. Therefore, we can presume that *GSTP1* Val114 polymorphism is associated with colorectal cancer susceptibility. Also, C allele confers protective effect to SCA while T allele increases the risk.[102, 110]

- ***GSTT* gene**

GSTT1 has a functional and a non-functional allele. Homozygosity for the nonfunctional allele of *GSTT1* is the *null* phenotype and exhibits an absence of catalytic activity. In our study, we observed that this polymorphism was prevalent in Portuguese population. However, this association is not consistent between all studies reported. Individuals homozygous for this deletion are thought to be at increased risk for malignancies (such as cancer, cardiovascular and respiratory diseases).[94, 95, 99, 101-104, 106] Similar to our data in SCA, several studies also reported that *GSTT1* polymorphism was significant for CRC risk in Caucasians.[95,101] Going into this, we observed a prevalence *GSTT1* null (75% vs 28%; p<0.0001; OR: 7.71; CI 3.83 to 15.56) genotype between SCA samples. Then, we can believe that subjects who carry *GSTT1* null polymorphism have an increased SCA risk. Whereas, those who have *GSTT1* functional genotype seems to have a lower SCA risk development.

- ***GSTM* gene**

Loss of GSTM enzyme function is described to a homozygous deletion of this gene resulting in the *GSTM1* null allele. Data have suggested that this polymorphism is associated with an increased risk of CRC, especially in the Caucasian population.[88, 89, 94-96, 99, 103-105,108] However, this research *GSTM1* del mutated genotype was not statistical significance between SCA patients and control group. Thus, we did not find a correlation of this polymorphism with SCA risk. There are some topics that can justify these findings. If in one way, GSTM1 have a low expression level in colon/rectum tissue (Figure 20), when compared with the other GSTs studied (Figure 21 and 22); in another way, GST are high polymorphic among ethnicities and we have not previous background of our population. Nevertheless, *GSTM1* null polymorphism has no association with SCA risk among our subjects.

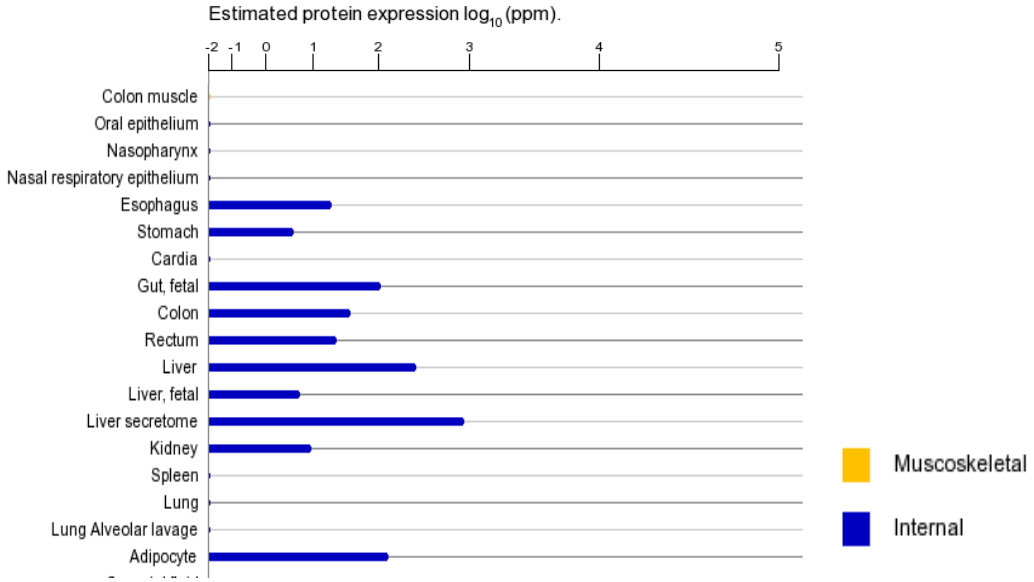


Figure 20: Protein expression of GSTM1 in different tissues.
Adapted from: http://cdn.genecards.org/images/v4/protein-expression/protein_expression_GSTM1.png

4.2. Gender

This cancer affects aged people in both sexes. As well other studies, there was no relation between any of the polymorphic variants and gender. We have no significant differences according to genotype distributions in patients. In our study, these results can be possibly explained by differences between sample genders distribution, since the majority of our samples are males (75% vs 25%). Nonetheless, it seems that most of polymorphisms distribution had no real difference among genders. In fact most studies reported no gender differences between CRC patients.

4.3. 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 oxidative stress and DNA damage. Rectal carcinomas show similar development mechanisms as in distal colon tumors (descending and sigmoid colon). Therefore, significant differences

between rectal and colon carcinomas with respect to genetic features have been reported.

SCA subjects in this study were stratified according to the place of primary tumor: colonic or rectal. Our results show that *GSTP1* 114T and *GSTP1* 114TT genotype have a significant difference between colon and rectum location on SCA patients. This difference could be explained by the GSTP1 high expression in colon which leads to a huge impact of these variants on this tissue when compared with rectum (Figure 21). Moreover, this polymorphism has been reported to have an important role in inflammatory process.[116,117] It is also known that inflammation could lead to colon cancer development, since most of colonic mucosa inflammatory diseases are associated with CRC risk.[118, 119,120,121] In this sense, *GSTP1* 114 mutation can influence colon inflammation progression and tumorigeneses process in colon. This association can explain the prevalence of this mutation in colon samples when compared with rectum. A previous study has also associated *GSTP* gene with liver tumorigenesis.[116]

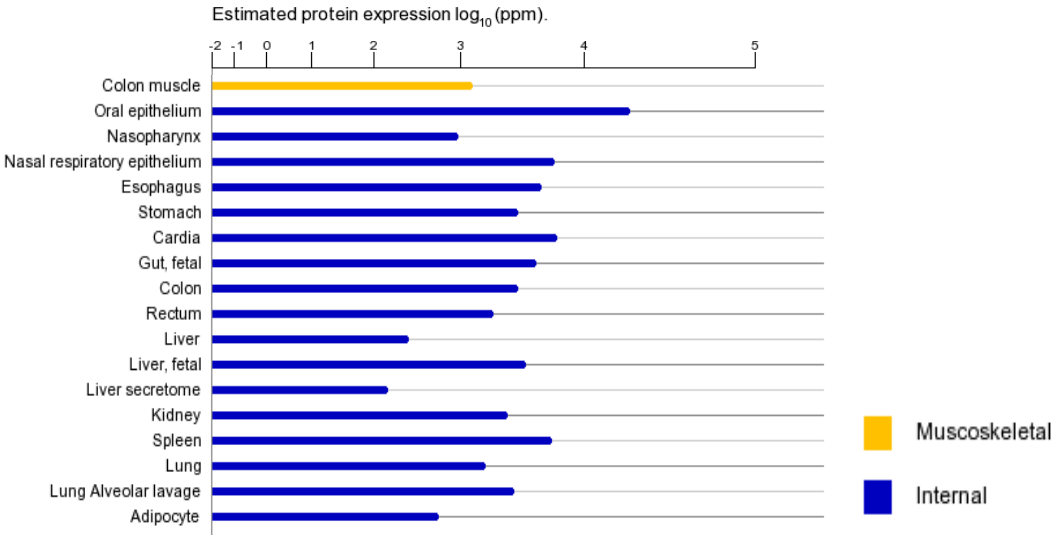


Figure 21: Protein expression of GSTP1 in different tissues.
Adapted from: http://cdn.genecards.org/images/v4/proteinexpression/protein_expression_GSTP1.png

Also, *GSTT1* null variant reveals significant differences in primer tumor location. Our results show that *GSTT1* null genotype has a higher prevalence in colon samples (87% vs. 64%). Like *GSTP1* 114T, this polymorphism has been

reported to have an important role in inflammatory process control in colon tissue.[121,122] *GSTT1* null genotype promotes colon inflammation progression and tumorigenesis process and both processes are involved in colon cancer development. These mechanisms can explain the prevalence of this mutation in colon samples when compared with rectum (Figure 22).

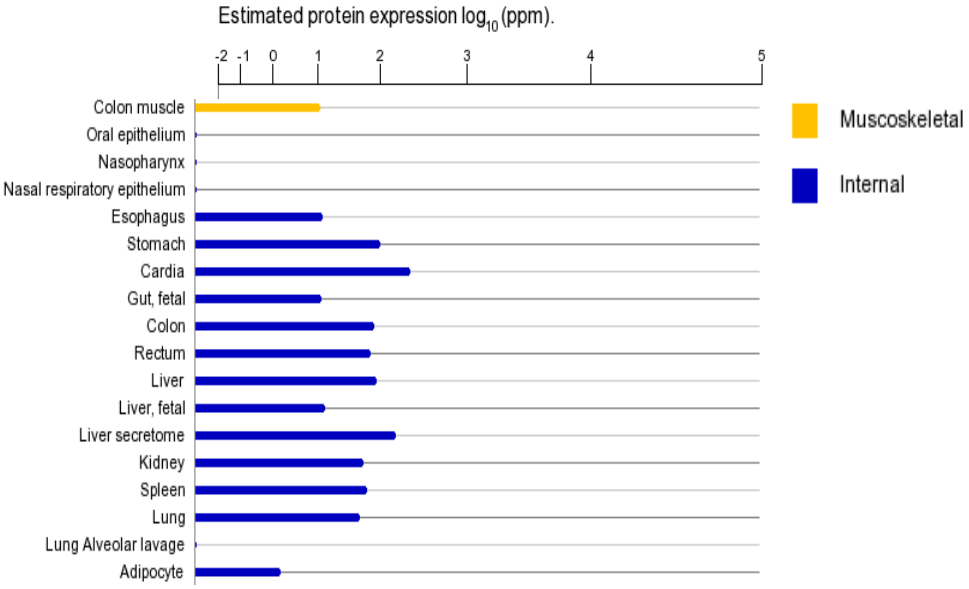


Figure 22: Protein expression of GSTT1 in different tissues.
Adapted from: http://cdn.genecards.org/images/v4/protein-expression/protein_expression_GSTT1.png

V. Conclusion

CRC is known as a multifactorial disease. Some diet and social habits, such as alcohol and smoking behaviors, represent a higher risk to CRC. However, the genetic influence is not fully understood. There have been reported that oxidative stress is involved in CRC development. Antioxidant and detoxify enzymes, namely SOD and GST, regulates ROS toxicity cellular levels and are crucial to this process. Moreover, polymorphic variants of those enzymes could lead to a redox hemostasis imbalance, particularly in colon and rectal tissues. In our work, we found that *MNSOD, SOD3, GSTP1* and *GSTT1* common polymorphisms were associated with increased SCA risk in Portuguese population. In normal conditions, they belong to a defense mechanism that regulates ROS levels. However, *MNSOD, SOD3, GSTP1* and *GSTT* genes polymorphisms encodes dysfunctional proteins. Therefore the defense mechanism will fail and ROS will accumulate, inducing an oxidative stress on colonic and rectal cells.

There may be others 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 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, lack of assess to patient's diet and habits). It is essential a large approach study with large sample size to confirm our outcomes. Still, the present study provides preliminary evidence that *MnSOD* T175C, *SOD3* R213G, *GSTP1* A105G, *GSTP1* C114T and *GSTT1* null polymorphisms, may be involved in SCA risk and could be useful to clarify this multifactorial disorder.

VI. Bibliography

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