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COX-2 POLYMORPHISMS IN COLORECTAL CARCINOGENESIS:

A STRATEGY FOR INDIVIDUALIZED CHEMOPREVENTION

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DISSERTAÇÃO DE MESTRADO DA LICENCIADA

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE MESTRE APRESENTADA À

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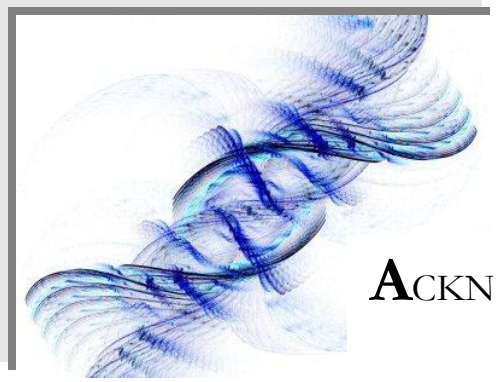
AOS MEUS PAIS POR NUNCA DESISTIREM…

…E POR ACREDITAREM EM MIM!



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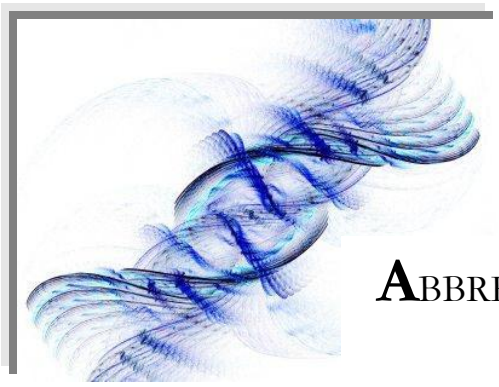


ABBREVIATIONS LIST

A

A – adenine

A - alanine

AA - arachidonic acid

AIM - atrophy and intestinal metaplasia

B

bFGF - basic fibroblast growth factor

BMI - body mass index

C

COX - cyclooxygenase

C - cytosine

CI - confidence interval

CRC - colorectal cancer

D

dbSNP – single nucleotide polymorphisms database

DM – dominant homozygous

DNA - deoxyribonucleic acid

df - degrees of freedom

E

EDTA - ethylenediamine tetraacetic acid

EGFR - epidermal growth factor receptor

ERK - extracelular signal-regulated kinase

F

FAP – familial adenomatous polyposis

G

G - glycine

G - guanine

GC - gastric cancer

GI – gastrointestinal

H

HWE - Hardy-Weinberg equilibrium

HNPCC – hereditary nonpolyposis colorectal cancer

I

I - isoleucine

IBD - inflammatory bowel diseases

L

L - leucine

M

M - methionine

MeSH - medical subject heading

mRNA - messenger ribonucleic acid

N

NCBI - national center for biotechnology information

NK - natural killer

NSAIDs - nonsteroidal anti-inflammatory drugs

O

OR - odds ratio

P

P - proline

PCR - polymerase chain reaction

PG – prostaglandins

PGDH - hidroxyprostaglandin dehydrogenase

PI3K - phosphoinositide-3 kinase

PPAR - peroxisome proliferator-activated receptor

PTGS - prostaglandin endoperoxidase synthase

R

R - arginine

RFLP - restriction fragment length polymorphism

S

SNP - single nucleotide polymorphism

SPSS - statistical package for social sciences

STROBE – strengthening the reporting of observational studies in epidemiology

T

T – thymine

U

UTR – untranslated region

V

V - valine

VAC – variant allele carriers

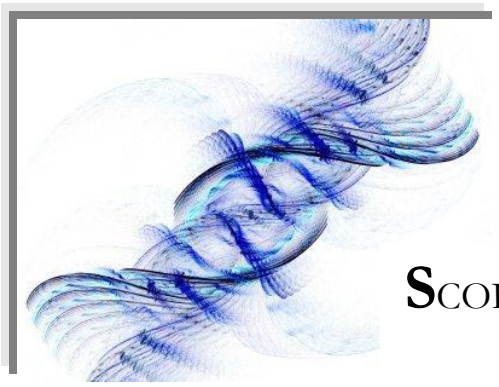
VEGF - vascular endothelial growth factor

W

w- tryptophan

WHO - world health organization

χ^2 - chi-square test



SCOPE & OUTLINE OF THE THESIS

The journey of the candidate in scientific research began with her undergraduate thesis on *COX-2* polymorphisms in cancer development, namely in gastric tumors, to obtain her degree in Applied Biology at University of Minho. She then was invited to continue her work, as a MSc student in this Medicine and Molecular Oncology Master Program (FMUP), at the Molecular Oncology Group and Gastroenterology Department of IPO Porto, in a wider perspective, including other polymorphisms in colorectal cancer. Following this line of investigation, the candidate has now been recently accepted as a PhD student at ICBAS-UP (last September 2008), to develop a thesis intended to provide a deeper knowledge on gastrointestinal (GI) carcinogenesis. It is our purpose to develop studies on 1) genetic variants of the arachidonic acid pathway as molecular markers with predictive value in GI tumors; 2) *in vitro* mechanistic and functional understanding of these variants; and 3) to go further, from bench to bed-side, and derivate models, that include as covariate these polymorphisms in order to select and implement both chemopreventive and management strategies in susceptible individuals. Therefore, a systematic review was performed to serve as the background upon which both a question for this Master thesis would be grounded and also the research questions for her PhD were programmed

This thesis is structured in **four** chapters:

In **chapter I**, the rationale for this study will be presented, focusing on the role of COX-2 enzyme and its inhibition in tumor development and the potential contribution of polymorphisms for colorectal cancer burden, followed by the statement of our hypothesis and aims.

A systematic review and meta-analysis on published studies addressing the contribution of *COX* polymorphisms in gastric and colorectal carcinogenesis, including the description of all *COX-2* polymorphisms previously analysed in colorectal tumors will be provided to readers in **chapter II**, as the background for the pursued research in the subsequent study.

In **chapter III,** a case-control study will be described and discussed to demonstrate as proof-of-concept, the impact of three specific *COX-2* polymorphisms, shown to modulate the susceptibility for colorectal tumors in the meta-analysis, in the development of CRC. To the best of our knowledge this is the first study to address the role of these polymorphisms in a gene-environment context in a Northern Portuguese population.

Finally, in **chapter IV** and considering the results of this study future research in this field of knowledge will be suggested.

The results presented on this thesis were published (chapter II) or submitted for publication (chapter III) as:

- Pereira C, Medeiros RM, Dinis-Ribeiro MJ. Cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis: are conclusive results available? *Eur J Gastroenterol Hepatol* 2009 Jan;21(1):76-91. There were some minor alterations made to the original paper content, most importantly the inclusion of a forest plot graphic reporting *COX-2* polymorphisms in CRC risk that for editorial reasons was excluded from the published version.
- Pereira C, Pimentel-Nunes P, Brandão C, Moreira-Dias L, Medeiros R, Dinis-Ribeiro M. *COX-2* Polymorphisms and Colorectal Cancer risk: A Strategy for Chemoprevention. (submitted for publication). The results presented on chapter III were also accepted for poster presentation at Digestive Disease Week 2009, Chicago, USA.

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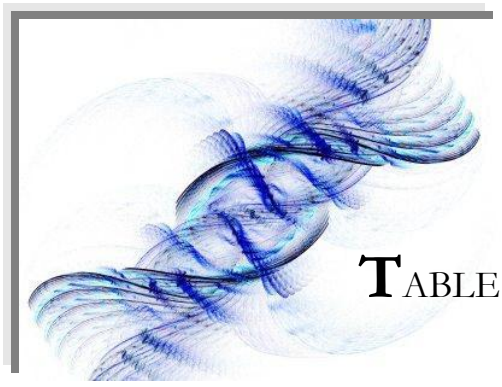

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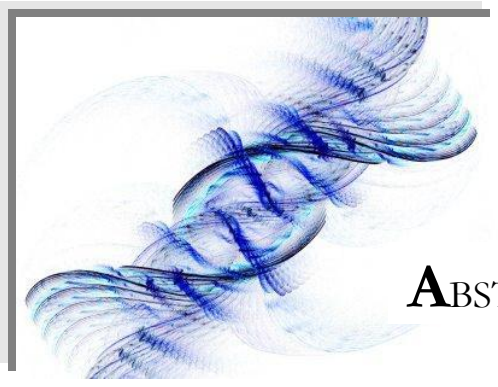


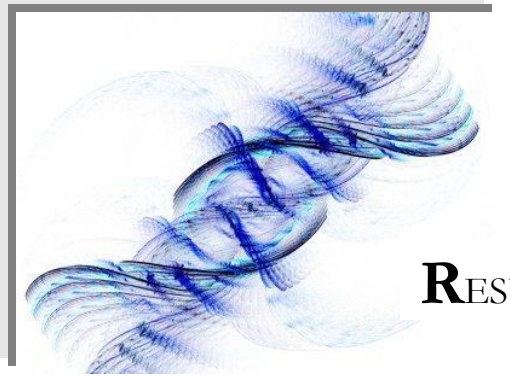
ABSTRACT

Colorectal cancer (CRC) is a major health concern ranking as the second leading malignancy with the highest incidence in Portugal in both genders. Nonsteroidal anti-inflammatory drugs (NSAID) where shown to exert their anti-inflammatory and anti-tumor activities through the suppression of cyclooxygenase (COX) enzymes. The inducible isoenzyme, COX-2, was found to be overexpressed in approximately 85% of colorectal adenocarcinomas, contributing to key steps in tumor development. The general aim of this thesis was to understand the influence of three *COX-2* polymorphisms, previously associated with the development of colorectal tumors as systematically reviewed, on the genetic susceptibility for CRC in a Northern Portuguese population.

We conducted a hospital-based case-control study involving 373 participants: 117 consecutively enrolled CRC patients and 256 healthy individuals without any clinical evidence of cancer. The -1329A>G, -899G>C and *429T>C *COX-2* polymorphisms were characterized through PCR-RFLP or Real-Time PCR allelic discrimination techniques.

Our findings revealed an interesting synergistic interaction between the -1329A>G polymorphism and smoke consumption in men that was translated in a 9-fold increased risk for CRC onset. Furthermore, these individuals, men ever-smokers carrying the -1329G allele had tan earlier onset of disease, with CRC diagnosis anticipated by seven years compared with -1329AA genotype carriers.

The -1329A>G *COX-2* polymorphism appears to modulate the genetic susceptibility for CRC onset, especially in men ever-smokers. This genetic profiling based higher-risk group definition may help shift the balance between risk and benefits for the use of COX-2 inhibitors in chemoprevention that is currently hampered by the adverse gastrointestinal and cardiovascular side effects.



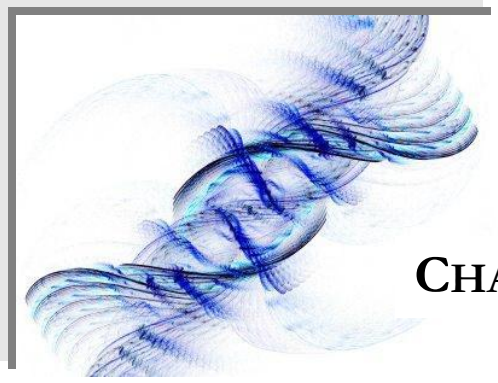
RESUMO

O cancro colo-rectal (CCR) assume-se como um sério problema de saúde pública, representando a segunda neoplasia com maior incidência em Portugal em ambos os géneros. Os anti-inflamatórios não esteróides (AINEs) devem as suas características, que os associam a um risco reduzido para tumores colo-rectais, à inibição da enzima COX-2. Esta enzima encontra-se sobre-expressa em 85% dos CCR, contribuindo para importantes etapas da carcinogénese. O objectivo geral desta tese focou, então, na análise da influência dos polimorfismos, -1329A>G, -899G>C e *429T>C no gene *COX-2*, que demonstramos através de uma revisão sistemática, modular o risco para tumores colo-rectais, na susceptibilidade genética para o desenvolvimento de CCR numa população do Norte de Portugal.

Foi desenvolvido um estudo caso-controlo de base hospitalar envolvendo 373 indivíduos: 117 com diagnóstico de CCR e 256 indivíduos sem evidência clínica de cancro. Os genótipos foram caracterizados por PCR-RFLP ou PCR em tempo real.

Os nossos resultados revelaram uma interacção entre o polimorfismo -1329A>G e o consumo de tabaco em homens, que se traduziu num aumento de 9x na predisposição para CCR. Adicionalmente, este indivíduos, homens fumadores ou ex-fumadores portadores do alelo -1329G viram o seu diagnóstico de CCR antecipado em 7 anos, comparativamente com os portadores do genótipo -1329AA.

Deste modo, a susceptibilidade genética para CCR parece ser modulada pelo polimorfismo -1329A>G, principalmente em indivíduos do sexo masculino que alguma vez tenham fumado. A incorporação de biomarcadores genéticos em modelos de risco para CCR poderá permitir um racional clínico para o uso de AINEs que actualmente se encontra comprometido pelas sérias complicações gastrointestinais que advêm do seu consumo prolongado.



CHAPTER I

RATIONALE & AIMS

"Cancer is to a large extent avoidable. Many cancers can be prevented. Others can be detected early in their development, treated and cured. Even with late stage cancer, the pain can be reduced, the progression of the cancer slowed, and patients and their families helped to cope." *World Health Organization*

1.1. COLORECTAL CANCER: THE PROBLEM…

Colorectal cancer (CRC) is a major health concern, representing the third most common and the fourth most frequent cause of cancer deaths worldwide.[1] Geographically, there is at least a 25-fold variation in CRC burden between countries with the highest incidences observed in developed countries,[2] as can be seen in Figure 1.

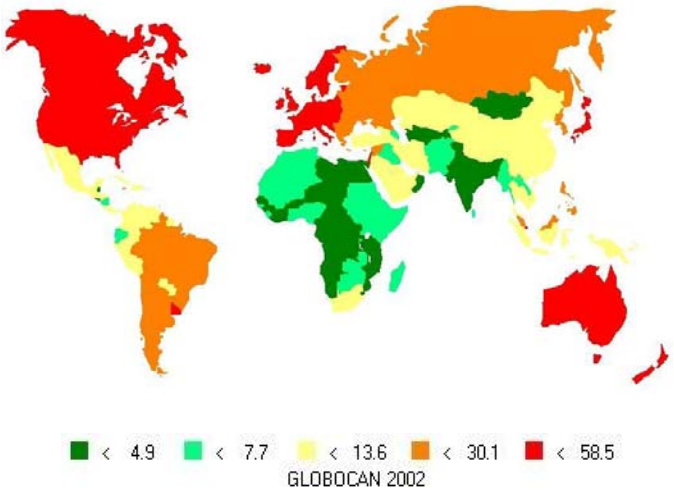


Figure 1. Incidence distribution of colorectal cancer among males in 2002 (per 100.000).[1]

The epidemiological picture in Portugal is similar, with CRC ranking as the second leading malignancy with the highest incidence in both genders, only behind prostate and breast cancers, in men and women, respectively.[1] Only in 2002, nearly 5-thousand persons developed CRC, over 13% of all diagnosed cancer cases.[1] Moreover, prognosis in CRC patients is highly dependable upon the stage of disease at diagnosis,[3] and although

screening has become a compelling strategy for prevention of colorectal tumors and shown to reduce CRC mortality,[4] only 40% of cases are diagnosed at earlier stages.[5] This calls for better compliance and improved CRC screening guidelines, not overlooking the potential impact of complementary chemopreventive approaches in reduction of this cancer burden.

1.2. ROLE OF COX-2 IN THE DEVELOPMENT OF COLORECTAL CANCER

Chronic inflammation and colorectal cancer are closely intertwined. In fact, inflammatory bowel diseases (IBD) such as, ulcerative colitis and Crohn's disease are well established conditions with increased predisposition for CRC.[6] Furthermore, drugs targeting inflammatory-related molecules, such as inducible nitric oxide synthase and cyclooxygenases (COX) were shown to exert anticancer properties with potential chemopreventive impact.[7,8]

There are at least two COX isoenzymes identified responsible for the biosynthesis of prostaglandins (PG) from arachidonic acid. COX-1 is a constitutive "housekeeping" enzyme ubiquitously expressed and associated with normal physiological functions.[9] Conversely, COX-2, the inducible isoenzyme, is normally absent in most tissues and shown to be up-regulated in 40-50% of adenomas and 85% of CRCs, as reported by Eberthart *et al.* in 1994.[10] Since then, intensive research has been developed to understand the role of COX-2 in tumor development. Briefly, COX-2-derived PGE_2, the most abundant prostaglandin in gastrointestinal tumors,[11] can promote carcinogenesis by activation of their cognate receptors (EP1-EP4)[12] and subsequently of several downstream signaling pathways that stimulate tumor cell proliferation, invasiveness and migration, enhance angiogenesis, inhibit apoptosis and modulate immunosupression,[13] as observed in Figure 2.

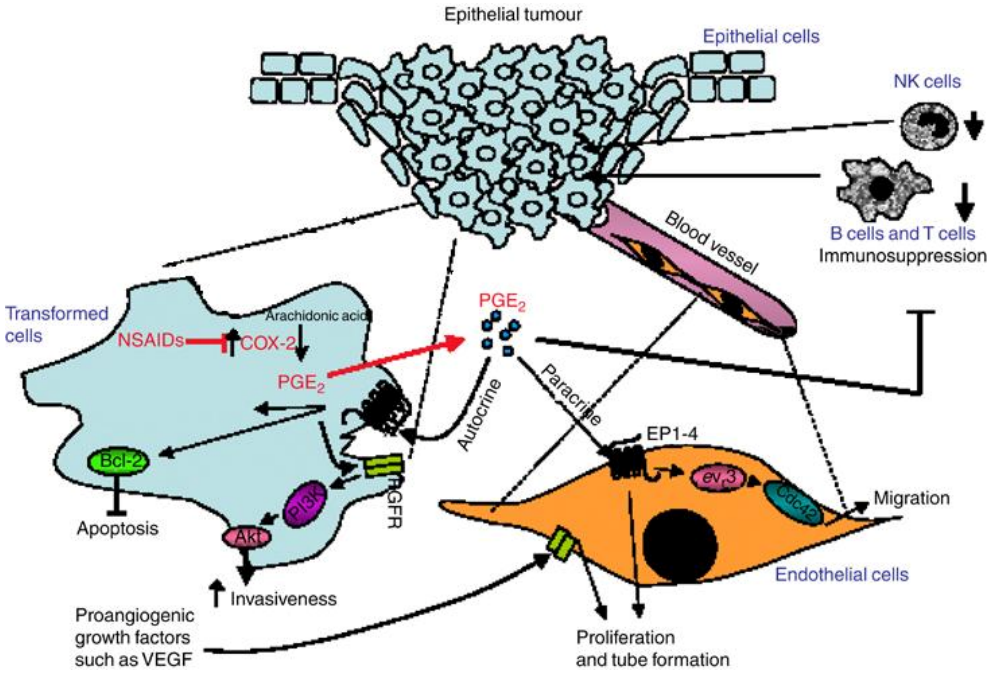


Figure 2. Mechanisms of COX-2-derived PGE_2 contribution to tumor development. Adapted from Wang *et al.*[14] In colorectal tumors, PGE_2, through both autocrine and paracrine regulation can: transactivate EGFR, which results in stimulation of cell migration and invasion through increased PI3K-Akt signaling; induce the production of angiogenic factors, such as, VEGF and bFGF that promote proliferation, migration and vascular tube formation; promote tumor survival by activating PPARδ via PI3K-Akt pathway and inducing antiapoptotic protein expression, like Bcl-2; and modulate immunosurveillance via inhibition of dendritic cell differentiation and T cell proliferation and suppression of antitumor activity of NK cells and macrophages, as reviewed by Wang *et al.*[13,15] bFGF, basic fibroblast growth factor; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; NK, natural killer; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE_2, prostaglandin E2; PI3K, phosphoinositide-3 kinase; PPAR, peroxisome proliferator-activated receptor; VEGF, vascular endothelial growth factor.

1.3. COX-2 AS A TARGET FOR COLORECTAL CANCER CHEMOPREVENTION

The rationale for the development of chemopreventive approaches in CRC prevention arose from observational studies highlighting the long-term use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) in the reduction of CRC incidence[16] and mortality[17]. The mechanism underlying the protective actions of NSAIDs is not completely understood but COX enzymes are their best known targets.[18]

In 1988, Kune *et al.*,[16] in the first population-based case-control study, reported a nearly 50% risk reduction for CRC development in regular aspirin consumers. Thereafter, a large body of epidemiological evidences consistently corroborated the protective role of NSAIDs in all stages of colorectal carcinogenesis. Recently, Flossmann and Rothwell[19] systematically reviewed relevant observational studies and analyzed the long-term effect of

aspirin in two randomized trials with more than 20 years of post-trial follow-up. In the clinical setting, allocation to 300 mg of aspirin daily for a minimum period of 5 years resulted in an effective primary prevention of CRC with a 10 years latency period that is similar to the effects observed in the observational studies.

Although effective, the prolonged use of NSAIDs is associated with serious gastrointestinal side-effects,[20] through COX-1 inhibition, that compromised their generalized use in a cost-effective approach.[21] However, and quoting Arder and Levin[22] "…*to ignore the potential benefit of chemoprevention is to continue to accept a higher than necessary death rate from CRC in patient populations that are not fully compliant with screening for colorectal neoplasia*".

A more comprehensive understanding of colorectal carcinogenesis, through the identification of genetic and environmental risk factors, might contribute to CRC prevention by targeting screening and chemoprevention to higher-risk individuals.

1.4. POLYMORPHISMS AS RISK FACTORS FOR COLORECTAL CANCER

Polymorphisms are common DNA genetic alterations present in at least 1% of the general population. Low-penetrance susceptibility alleles are defined as polymorphic genes with specific alleles that are associated with an altered risk for disease susceptibility.[23]

Genetic predisposition certainly has a key role in CRC development. In a large study in twin it was noticed that heritability contributes to 35% of all CRC cases.[24] Moreover, almost 20% of all patients have a positive familial history,[25] while only 5-10% fulfill the criteria for hereditary colorectal cancer, suggesting the involvement of low-penetrance alleles for the remaining familiality.[26] Although, germline mutations in key genes associated with hereditary syndromes have an extremely high CRC penetrance, the fraction of cases attributable to these disorders is somewhat small, as mentioned. Whereas, the contribution of low-penetrance alleles, normally associated with modest risk estimates, to the total CRC burden could be relatively high, explained by their common frequency.[26] Hence, it is reasonable to expect that some polymorphisms in *COX-2* gene might modulate the

susceptibility for CRC, thereby, yielding the recognition of individuals at higher risk for this disease.

1.5. GENERAL AIM

The general aim of this thesis was to evaluate the association of *COX-2* polymorphisms to the development of colorectal cancer.

1.5.1. Specific Aims

- To evaluate the influence of -1329A>G, -899G>C and *429T>C polymorphisms in *COX-2* in the risk and time to onset of CRC in a Northern Portuguese population.
- To measure possible gene-environment interactions between *COX-2* polymorphisms and other known risk factors for CRC.

1.6. REFERENCES

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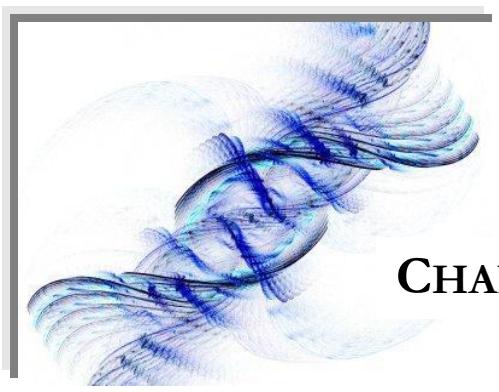

CHAPTER II

BACKGROUND: A SYSTEMATIC REVIEW ON THE ROLE OF *COX* POLYMORPHISMS IN GASTROINTESTINAL TUMORS

2.1. INTRODUCTION

Gastrointestinal cancers are amongst the leading causes of cancer mortality. Gastric and colorectal cancers (CRC), with the second and fourth highest cancer mortalities, respectively, were responsible for 1 229 327 deaths, approximately 18.3% of all cancer-related mortality in 2002.[1]

Several types of studies demonstrated that gastric and colorectal carcinogenesis develops upon a multistep process involving the transformation of normal mucosa to benign precancerous lesions, such as atrophy and intestinal metaplasia (AIM) to gastric adenocarcinoma (GC) (according to Correa's model),[2] and adenomatous polyps to CRC.[3,4] Furthermore, these cancers are complex and multifactorial diseases, emerging from the combined influence of environmental factors such as diet, lifestyle, Helicobacter pylori infection, and the individual genetic background. [5-8]

Inflammation, nowadays, is a field of highlighted interest in the development and progression of several gastrointestinal cancers.[9-11] Epidemiological and animal data revealed that the use of NSAIDs might reduce the risk of gastric cancer, colorectal polyps, and cancer.[12-15] Although not clearly understood, the ability of NSAIDs to suppress inflammation is strongly credited to the inhibition of cyclooxygenase (COX) enzyme.[16,17]

COXs or prostaglandin endoperoxide synthases (PTGS) are rate-limiting enzymes that catalyze the formation of prostaglandins (PG) from arachidonic acid (AA).[18] COX-1 is constitutively expressed in the majority of tissues and is associated with housekeeping functions like vascular homeostasis and platelet aggregation.[19] The second isoform (COX-2), almost undetectable under normal physiological conditions, is readily induced in response to mitogens, tumor promoters, cytokines, growth factors, stress-inducing agents promoting inflammatory reactions, and tumor development.[9–21] COX-2 overexpression was reported in several common human malignancies, predominantly of the gastrointestinal tract,[22,23] including in 85% of CRC and 67% of GC tumors also as in their associated precancerous lesions.[24,25]

Therefore, it is not surprising that functional polymorphisms in *COXs* genes have been studied to define their influence in the susceptibility to develop gastrointestinal malignant diseases. The discovery of single nucleotide polymorphisms (SNPs) as potential biomarkers in early gastrointestinal tumorigenesis[26] has prompted the development of several studies that are often limited by the small statistical power, hindering a clear definition of the impact of those polymorphisms in gastric and CRC development. Therefore, we conducted a systematic review to unravel the influence of *COX* polymorphisms in gastrointestinal cancers and associated lesions. This could ultimately allow the recognition of individuals at higher risk that may benefit from surveillance programs and/or chemopreventive strategies.

2.2. MATERIALS AND METHODS

2.2.1. Type of study

A systematic review was conducted on manuscripts obtained after applying the inclusion and exclusion criteria (see below) to abstracts collected by introducing a specific query in an on line database (PubMed). Selected studies were then characterized in a structured sheet, the quality assessed and the pooled data statistically analyzed.

2.2.2. Search strategy and papers selection

A MEDLINE database (PubMed) search was performed to retrieve papers linking *COX* polymorphisms and risk of gastrointestinal cancers available on line by May 2008, using the following query: *'{[cox OR cox1 OR cox2 OR ptgs1 OR ptgs2 OR 'Cyclooxygenase 2'(MeSH) OR 'Cyclooxygenase 1'(MeSH)] AND [polymorphism OR polymorphisms OR 'Polymorphism, Genetic'(MeSH) OR 'Polymorphism, Single Nucleotide'(MeSH)]} AND {['gastrointestinal cancers'(All Fields) OR 'Gastrointestinal Neoplasms' (MeSH) OR 'Digestive System Neoplasms'(MeSH)] OR*

[gastric OR 'Stomach' (MeSH)] AND [cancer OR 'Neoplasms'(MeSH) OR 'precancerous lesions' OR 'Precancerous Conditions'(MeSH) OR adenocarcinoma OR atrophy OR dysplasia OR 'Polyps'(MeSH) OR 'Adenomatous Polyps'(MeSH)]} OR 'intestinal metaplasia' OR 'Gastritis, Atrophic'(MeSH) OR {['Esophagus'(MeSH) OR esophageal] AND [cancer OR 'Neoplasms'(MeSH) OR adenocarcinoma OR 'squamous cell carcinoma']} OR 'barrett syndrome'(All Fields) OR 'Barrett Esophagus'(MeSH) OR {['Colon'(MeSH) OR colonic OR colorectal OR intestinal OR bowel OR rectal] AND [cancer OR 'Neoplasms'(MeSH) OR 'precancerous lesions' OR 'Precancerous Conditions'(MeSH) OR adenocarcinoma OR 'Polyps'(MeSH) OR 'Adenomatous Polyps'(MeSH)]}'.

One hundred and fifty-one abstracts gathered through this search were then read and the inclusion/exclusion criteria applied independently by two researchers (PC and DRM) (Figure 1).

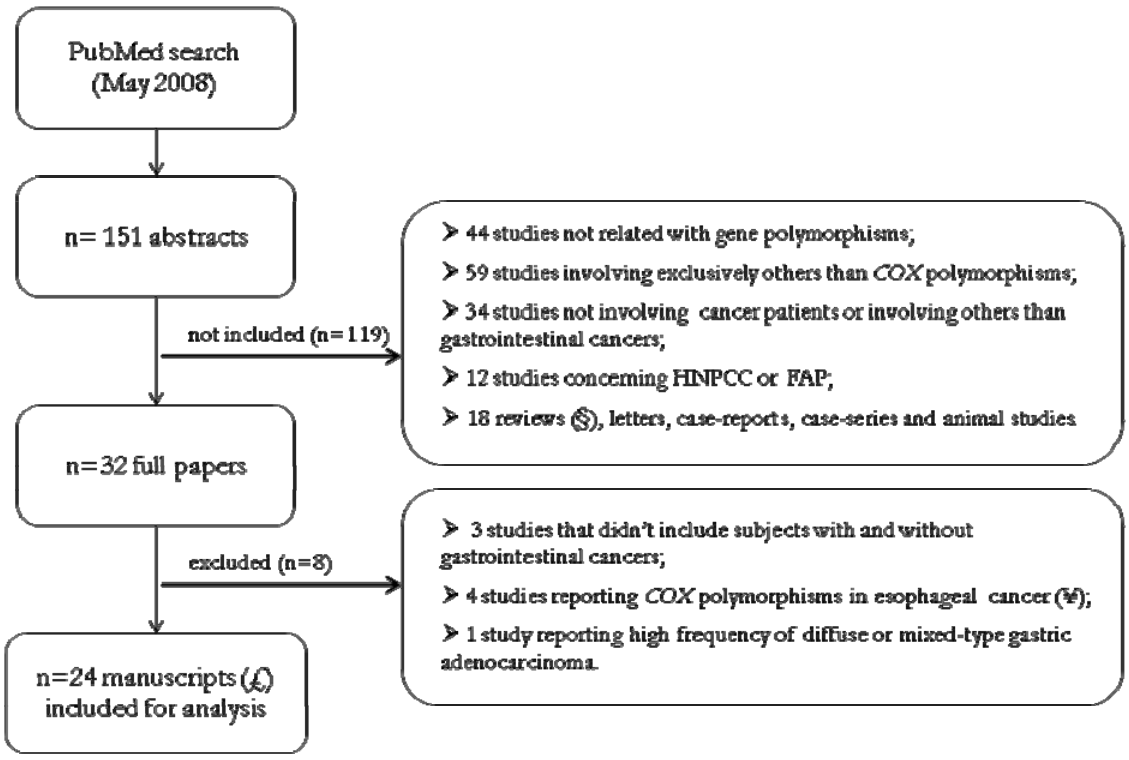

Figure 1. Fluxogram for retrieval and paper selection. §, the reference list from reviews was checked for missing papers in the PubMed search. No additional studies were found. ¥, four studies involving esophageal cancer were omitted: two with overlapping data on squamous cell carcinoma[27,28] and other two studies focusing on esophageal adenocarcinoma examined different *COX-2* polymorphisms;[29,30] £, one study was reported in Chinese without Portuguese, English, French or Spanish version.[31] It was considered in the statistical analysis; FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colorectal cancer.

Observational studies (case–control and cohort) aimed at assessing the association between *COX* polymorphisms and sporadic gastrointestinal cancers and/or its precancerous lesions were included in this systematic review.

Of the 32 articles meeting the primary criteria,[27-58] seven were promptly excluded: four studies investigating esophageal cancer were omitted (two with overlapping data on esophageal squamous cell carcinoma[27,28] and the two studies focusing on esophageal adenocarcinoma examined different *COX-2* polymorphims[29,30]); the other three did not include patients with and without gastrointestinal lesions in their studies [i.e. not allowing the odds ratio (OR) estimation].[33,34,58] We also retrieved one paper published in Chinese without English, French, Spanish, or Portuguese version.[31] To overcome this language barrier, a genotype distribution extraction sheet was sent, by e-mail, to the authors. After exploring full papers another report was excluded. In the study developed by Sitarz *et al.*[57] an extremely high frequency of diffuse and mixed-type GC was noticed (over 60%) and as COX-2 is mainly associated with the intestinal histological-type multistep cascade,[59] considering this study in the pooled analysis could disguise the real impact of *COX-2* naturally occurring genetic variations in gastric carcinogenesis.

Finally, the reference list of all selected publications and review articles excluded was also checked for additional studies missed on the PubMed search, although no further studies were included.

2.2.3. Data extraction and quality assessment

From each of the included articles the following information was extracted: first author, year of publication, country, ethnicity, study design, histological type, gene and polymorphisms analyzed, number of cases and controls by genotype, representativeness of cases, source of controls, histopathological confirmation of cases and controls, genotyping examination, confounding factors, variables used in statistical adjustments, and evidence of Hardy–Weinberg equilibrium (HWE). If an article reported results from different study

populations, those populations were assessed independently.[42,47] From the studies by Koh *et al.*[49] and Tan *et al.*[44] only the pooled CRC's genotype frequencies were extracted, although they had data concerning colon and rectal cancer independently.

Furthermore, two overlapping reports were found from Siezen *et al.*[32,40] Likewise, the paper written by Poole *et al.*[56] had duplicated data with both studies from Ulrich *et al.*[37,38] All articles were included for quality assessment, but only the more recent and/or complete article was incorporated in the pooled data.[37,38,40] Articles with stratified results by gender were pooled together.[39,50] Data regarding the use of NSAIDs were assessed either independently or combined.[37,38,40,42]

The quality of papers was also independently assessed by two researchers (MR and DRM) based on two published quality score systems, one created by Thakkinstian *et al.*[60] and the STROBE statement[61] (Addenda 1). Scores ranged from 0 (lowest) to 50 (highest).

2.2.4 Polymorphism characterization

In this systematic review, the different *COX* polymorphisms were designated following the nomenclature proposed by den Dunnen and Antonarakis in 2001.[62] The different polymorphisms are characterized in Addenda 2.

Some ambiguity across studies when defining the variant allele for the -1329G>A *COX-2* polymorphism was observed. Therefore, considering the functional study developed by Zhang *et al.*[27] we defined the A allele as the variant one since it was shown to increase *COX-2* transcriptional activity.

2.2.5 Statistical analysis

Pooled variant allele frequencies in control populations

The pooled frequency of each *COX* polymorphism, stratified by ethnicity, was estimated only in the control populations. Heterogeneity across studies was measured through the χ^2 test [degrees of freedom (df) equal to the number of studies minus one].

Hardy–Weinberg equilibrium assessment in control populations

Before the effect estimation of the several *COX* polymorphisms in gastric and colorectal carcinogenesis, the HWE was assessed for all the polymorphisms in each study, whenever unavailable in the original papers, by using the χ^2 test or Fisher's exact test, where appropriate (n<5) (1df). If *P* value was less than 0.05 then control genotype distributions were assumed to deviate from HWE.

Estimated effect of cyclooxygenase polymorphisms in gastric and colorectal carcinogenesis

For both individual or pooled OR and the corresponding 95% confidence interval (95% CI) estimation, data was inserted on RevMan 4.2.10 statistical program (Copenhage, Denmark).[63] The dominant model was assumed for every polymorphism and the ORs were estimated under a random-effect model.[64] The heterogeneity statistics was based on Q-value that follows a χ^2 distribution (df equal to the number of studies minus one).[65] When *P* value was under 0.05, a statistically significant heterogeneity was assumed among studies. Sources of heterogeneity were appraised by subgroup stratification, based on several study characteristics, like ethnicity and source of control individuals (population or hospital based).

The influence of *COX-1* and *COX-2* polymorphisms in gastric and colorectal carcinogenesis was assessed independently for each of the following groups: the gastric precancerous lesions group that included patients with AIM;[35,36,53] the gastric cancer patients with lesions as severe as low-grade dysplasia,[31,35,36,52,53,55] excluding those with lesions indefinite for dysplasia from the study by Liu *et al.*;[36] the colorectal adenoma group,[37-42,54] disregarding patients with hyperplastic polyps reported in both studies by Ulrich *et al.*[37,38] and finally, the CRC group.[42–48,51]

Sensitivity analysis and publications bias

By including and excluding studies deviating from HWE sensitivity analysis was performed. Publication bias was assessed through funnel plot asymmetry tests.[66]

2.3. RESULTS

2.3.1. Description of studies

Table 1 summarizes all studies included in this meta-analysis. There were 24 manuscripts[31,32,35–56] reporting 22 studies[31,35–55] included in this review: (i) six studies addressed gastric carcinogenesis,[31,35,36,52,53,55] three of which had information not only characterizing gastric cancer patients but also a group with precancerous lesions[35,36,53] and (ii) 16 studies evaluated the risk of adenoma (n=6)[37–41,54] or CRC (n=9)[43–51] or had data regarding both (n=1).[42]

Nine *COX-1* polymorphisms were addressed to uncover their role in the development of gastrointestinal tumors: IVS7+14delA (rs3215925), IVS7-45T>C (rs3842798), Q41Q (rs3842788), G213G (rs5788), L15_L16del, R8W (rs1236913), P17L (rs3842787), L237M (rs5789) and V481I (rs5794). In *COX-2*, 17 polymorphisms were characterized: -1462_-1461delTG (rs689464), -1423A>G (rs689465), -1329G>A (rs689466), -899G>C (rs20417), -798A>G, -646C>T (rs20420), -196C>G (rs20424), -125T>G (rs5721), V511A (rs5273), IVS5-275T>G (rs20432), IVS7+111T>C (rs4648276), V102V (rs5277), G587R (rs3218625), *429T>C (rs5275), *1806A>G (rs4648298), *2291G>A (rs689469), *2430C>T (rs689470).

Table 1. Study Characterization by histological type.

Study [ref]	Country	Ethnicity	Design	Quality (max 50)	N¥	Representativeness of cases	Source of Controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Gastric Precancerous Lesions (Atrophy or Intestinal Metaplasia)										
Pereira, 2006 [31]	Portugal	*Caucasians*	Case-control	33	247	Hospital	Blood donors	All cases (EMB)	*COX-2* (-899G>C)	*Adjustment* for age and gender
Liu, 2006 [32]	China	*Asians*	Case-control	33	841	Population	Population (SG/CAG)	All controls and cases (EMB)	*COX-2* (-899G>C, -1329G>A, G587R)	*Adjustment* and *Stratification* for age, gender, *H. pylori* infection, smoking, alcohol drinking
Canzian, 2008 [50]	Venezuela	*Hispanics*	Case-control	39	1863	Population	Population (including CG)	All controls and cases (EMB)	*COX-1* (Q41Q, G213G, L237M, V481I, IVS7+14delA, IVS7-45T>C); *COX-2* (IVS5-275T>G, V102V, *429T>C)	*Adjustment* for age, gender, *H. pylori* infection, smoking, fruit and starchy intake and other
Gastric Adenocarcinoma										
Pereira, 2006 [31]	Portugal	*Caucasians*	Case-control	33	283	Hospital	Blood donors	All cases (EMB)	*COX-2* (-899G>C)	*Adjustment* for age and gender
Liu, 2006 [32]	China	*Asians*	Case-control	33	816	Population & Hospital	Population	All controls and cases (EMB)	*COX-2* (-899G>C, -1329G>A, G587R)	*Adjustment* and *Stratification* for age, gender, *H. pylori* infection, smoking, alcohol drinking
Zhang, 2006 [33]§	China	*Asians*	Case-control	-	969	-	-	-	*COX-2* (-1423A>G, -1329G>A, -899G>C)	-
Saxena, 2008 [49]	India	*Indians*	Case-control	34	303	Hospital	Hospital (NUD)	All controls and cases (E)	*COX-2* (-899G>C)	*Adjustment* for age and gender; *Stratification* for *H. pylori* infection
Canzian, 2008 [50]	Venezuela	*Hispanics*	Case-control	39	1169	Population (Dys)	Population	All controls and cases (EMB)	*COX-1* (Q41Q, G213G, L237M, V481I, IVS7+14delA, IVS7-45T>C); *COX-2* (IVS5-275T>G, V102V, *429T>C)	*Adjustment* for age, gender, *H. pylori* infection, smoking, fruit and starchy intake and other
Hou, 2007 [52]	Poland	*Caucasians*	Case-control	33	732	Hospital	Population	All cases (nt)	*COX-1* (L237M, V481I); *COX-2* (-899G>C, IVS5-275T>G, IVS7+111T>C, V102V, *429T>C, *2430C>T)	*Matching* for age and gender; *Adjustment* for age, gender, smoking, family history of cancer, diet

Table 1. Study Characterization by histological type (continued).

Study	Country	Ethnicity	Design	Quality (max 50)	N¥	Representativeness of cases	Source of Controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Colon Adenoma										
Ulrich, 2004 [34]†	USA	*Caucasians*	Case-control	36	1142	Hospital	Hospital	All controls and cases (C)	*COX-1* (R8W, L15-L16del, P17L, L237M)	*Adjustment* for age, gender, BMI, ethnicity, physical activity, smoking, diet and other; *Stratification* for NSAIDs use
Ulrich, 2005 [35]†	USA	*Caucasians*	Case-control	36	1078	Hospital	Hospital	All controls and cases (C)	*COX-2* (-899G>C)	*Adjustment* for age, gender, BMI, alcohol drinking, smoking, diet; *Stratification* for NSAIDs use
Ali, 2005 [36]	USA	*Caucasians*	Nested case-control	40	1505	Population	Population	All controls and cases (S)	*COX-2* (-798A>G, IVS5-275T>G, *429T>C, -1462_-1461delTG)	*Matching* for gender; *Adjustment* for age, gender, smoking and NSAID use; *Stratification* for gender, smoking and NSAIDs use
Siezen, 2005 [27]‡	Netherlands	*Caucasians*	Case-control	37	787	Hospital	Hospital	All controls and cases (E)	*COX-1* (R8W, L237M); *COX-2* (-1329A>G, V102V, *429T>C	*Adjustment* for age, gender, alcohol drinking; *stratification* for diet
Siezen, 2006 [37] ‡#	Netherlands	*Caucasians*	Case-control	37	784	Hospital	Hospital	All cases and controls (E)	*COX-1* (R8W, L237M); *COX-2* (-1329A>G, -899G>C, V102V, *429T>C)	*Matching* for age, gender and setting; *Adjustment* for age, gender, duration of smoking; *Stratification* for gender, smoking, family history of cancer, total and fatty fish intake
Gunter, 2006 [38]#	USA	*Caucasians*	Case-control	38	475	Hospital	Hospital	All controls (S) and all cases (S or C)	*COX-2* (-899G>C, V102V, IVS5-275T>G, *429T>C)	*Matching* for age and gender; *Adjustment* for age, gender, ethnicity
Lin, 2002 [39]	USA	*African-Americans*	Case-control	35	240[(A)]/ 140[(B)]	Hospital	Hospital	All controls and cases [(A)] (FS) and All controls and cases [(B)] (C)	*COX-2* (V511A)	*Matching* for age, gender, and setting; *Adjustment* for age and gender; *Stratification* by NSAIDs use
Ueda, 2008 [51]	Japan	*Asians* (men)	Case-control	39	1507	Hospital	Hospital	All controls and cases (C)	*COX-2* (-1329G>A, -899G>C, *1806A>G)	*Adjustment* for age, alcohol drinking, smoking BMI, setting

Table 1. Study Characterization by histological type (continued).

Study [ref]	Country	Ethnicity	Design	Quality (max 50)	N¥	Representativeness of cases	Source of Controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Poole, 2007 [53]†	USA	*Caucasians*	Case-control	37	1114	Hospital	Hospital	All controls and cases (C)	*COX-1* (R8W, P17L); *COX-2* (-899G>C)	*Adjustment* for age, gender, BMI, ethnicity, alcohol drinking, smoking, diet, NSAIDs use; *Stratification* for fish intake
Colon Adenocarcinoma										
Hamajima, 2001 [40]	Japan	*Asians*	Case-control		389	Hospital	Hospital	All controls (E) and cases (nt)	*COX-2* (-899G>C, -125T>C, -163C>G)	
Tan, 2007 [41]	China	*Asians*	Case-control	30	2300	Hospital	Population	All controls (E) and cases (CP or E)	*COX-2* (-899G>C, -1329A>G, -1423A>G)	*Adjustment* for age and gender
Cox, 2004 [42]	Spain	*Caucasians*	Case-control	35	566	Hospital	Hospital	All cases (nt)	*COX-2* (-899G>C, V102V, IVS5-275T>G, *429T>C, -1423A>G, *1806A>G, -196C>G, *2291G>A)	*Matching* for age and gender; *Adjustment* for age and gender
Landi, 2006 [43]	Spain	*Caucasians*	Case-control	24	703	Hospital	Hospital	nt	*COX-1* (V481I)	*Adjustment* for age and gender
Goodman, 2004 [44]	USA	*Caucasians and African-Americans*	Case-control	30	511 (D)/ 315 (E)	Hospital	Hospital & Population	nt	*COX-1* (L237M, V481I); *COX-2* (-646C>T, V511A)	*Adjustment* for age, gender; *Stratification* for ethnicity
Siezen, 2006b [45]	Netherlands	*Caucasians*	Nested case-control	37	603	Population (PPHV)	Population (PPHV)	nt	*COX-1* (R8W, L237M); *COX-2* (-1329A>G, V102V, *429T>C, *1806A>G)	*Matching* for age, gender and setting; *Adjustment* for age, gender, smoking
Koh, 2004 [46]	China	*Asians*	Nested case-control	38	1487	Population	Population	All cases, except three (ascertained by death records and clinical evidence)	*COX-2* (-899G>C)	*Adjustment* for age, gender, BMI, smoking, alcohol drinking, familial history of colorectal cancer; *Stratification* by diet
Lin, 2002 [39]	USA	*African-Americans*	Nested Case-control	35	396	Population	Population	nt	*COX-2* (V511A)	*Adjustment* for age and gender; *Stratification* by NSAIDs use

Table 1. Study Characterization by histological type (continued).

Study	Country	Ethnicity	Design	Quality (max 50)	N¥	Representativeness of cases	Source of Controls	Histopathological or endoscopic confirmation	Gene (polymorphisms)	Main variables addressed
Sansbury, 2006 [47] #	USA	*African-Americans*	Case-control	31	566	Population	Population	nt	*COX-2* (V511A)	*Matching* by age and gender; *Adjustment* for age, gender, offset term; *Stratification* for NSAIDs use
Xing, 2008 [48]	China	*Asians*	Case-control	30	336	Hospital	Hospital	All cases (nt)	*COX-2* (-899G>C)	*Adjustment* for age, gender, smoking, alcohol drinking and BMI; *Stratification* for smoking, alcohol drinking and BMI

¥, number of individuals studied; §, from this study by Zhang *et al.*[33] written in Chinese without English, French, Spanish or Portuguese version we only extracted the genotype distribution and 95% CI OR estimates for all polymorphisms (through e-mail contact with authors). The other informations were extracted directly from the abstract; †, overlapping data. Only the studies by Ulrich *et al.*[34,35] were comprised in this study (higher number of individuals genotyped); ‡, duplicated data. Only the more recent and complete study[37] was included in the meta-analysis. #, supplemental informations regarding study description was collected from the cited references; (A), in USC/Kaiser study; (B), in UNC study; (D), Caucasian population; (E), African-American population; Dys, dysplasia; SG/CAG, superficial gastritis / chronic atrophic gastritis; CG, chronic gastritis; NUD, non ulcer dyspepsia; PPHV, Project on Cardiovascular Disease Risk Factors; EMB, Endoscopic Multiple Biopsies; C, Colonoscopy; S, Sigmoidoscopy; E, endoscopy; FS, flexible sigmoidoscopy; CP, coloprotectomy; nt, method not mentioned; BMI, body mass index; NSAIDs, nonsteroidal anti-Inflammatory drugs.

2.3.2. Studies' design

All studies were considered as case–controls, including the four nested case–controls.[39,42,48,49] The median quality was 34.4 points (ranging from 24[46] to 40[39]).

Twelve studies used hospital-based cases and controls[35,37,38,40,41,43,45–47,51,52,54] and eight described a population-based design.[36,39,44,48–50,53,55] In the study by Lin *et al.*[42] the risk assessment for colorectal adenoma and cancer pursued a hospital an population-based design, respectively. Only seven studies reported case and control group matching, at least, for age and gender.[40–42,45,48,50,55] All studies adjusted their data for potential confounders.[35–42,44–55] Twelve studies reported the endoscopic and/or histological confirmation of the cancer-free status for the control groups.[36–44,52–54] Cases and controls were mainly defined through endoscopy procedures, with histological assessment.

2.3.3. Number of participants and addressed populations

A total of 20576 individuals were studied: 2951 within the precancerous gastric lesions group analysis; 4272 in the gastric carcinoma; 6871 in the colorectal adenoma, and 8172 in the colon cancer groups. Globally, the median number of individuals included was 653, with a minimum of 140[42] and an upper limit of 2300 participants in the study by Tan *et al.*[44]

Five ethnicities were addressed: eleven studies focused on Caucasian populations,[35,37–41,45–48,55] seven on Asiatic,[31,36,43,44,49,51,54] three on African–American,[42,47,50] one on a Hispanic,[53] and one on a Northern Indian population.[52] The African–Americans were only regarded in colon tumors, whereas the Hispanics and an adult population from Northern Indian were exclusively analyzed for onset of gastric lesions.

2.3.4. Allele frequencies in control populations

The genotype distribution of *COX* polymorphisms following the dominant model and the variant allele frequency is described in Tables 2–5. Briefly, there was homogeneity among study populations for all *COX-1* polymorphisms, except for the L237M between Caucasians (P=0.012). The A allele frequency ranged from 2[40,47,55] to 4%[48]. In *COX-2*,

heterogeneity was detected across the six studies evaluating the -899G>C SNP in Caucasians (*P*=0.022) and the pooled C allele frequency ranged from 13[40] to 22%[35]. Likewise, the six Asiatic studies reporting this polymorphism were also heterogeneous (*P*<0.001) (2[31,43,44] to 8%[51]). The *429T>C genetic variation also showed heterogeneity among Caucasians (*P*<0.001).[39–41,45,48,55] The study developed by Gunter *et al.*[41] revealed a *429C allele distribution outside the ones observed in the other five studies (65% vs. 30–36%, *P*<0.001). The -1423A>G and -1329G>A were detected at a higher frequency among Caucasians than in Asiatic populations (17[45] vs. 5%[31,44], for -1423G allele, *P*<0.001 and 79[40,48] versus 51%[31,36,44,54] for -1329A allele, *P*<0.001). With the exception of V511A[47] and -646C>G[47] *COX-2* SNPs in Caucasians, and *1806A>G in a Japanese population,[54] all the other genetic variations are present, by definition, in at least 1% of the population.[67] Through the different studies, all polymorphisms were in HWE but the IVS5-275T>G *COX-2* SNP characterized by Ali *et al.*[39]

2.3.5. Gastric carcinogenesis and *COX* polymorphisms

Fifteen COX polymorphisms were assessed for their role in gastric carcinogenesis. In Tables 2 and 3, the random-effect ORs following the dominant genetic model for each polymorphism on the risk of gastric lesions is summarized. No publication bias was evident.

Risk of gastric precancerous lesions

Estimated risk of *COX-1* polymorphisms

Only the study by Canzian *et al.*[53] reported the involvement of *COX-1* polymorphisms in AIM development. No statistically significant association was observed for any of the six polymorphisms.

Table 2. Random-effects *Odds Ratio* (unadjusted) and 95%CI estimated in this analysis following the dominant model of inheritance for gastric precancerous lesions onset.

Polymorphism	First author, year [ref]	Cases (%)		Controls (%)		Variant allele frequency	OR (95% CI)
		DH	VAC	DH	VAC		
COX-1							
Q41Q (A>G)	Canzian, 2008 [50]†	752 (93)	53 (7)	957 (92)	84 (8)	0.04	0.80 (0.56-1.15)
G213G (C>A)	Canzian, 2008 [50]	471 (59)	333 (41)	613 (58)	439 (42)	0.24	0.99 (0.82-1.19)
L237M (C>A)	Canzian, 2008 [50]†	776 (96)	32 (4)	1020 (97)	33 (3)	0.02	1.27 (0.78-2.09)
V481I (G>A)	Canzian, 2008 [50]†	798 (98)	12 (2)	1038 (99)	12 (1)	0.01	1.30 (0.58-2.91)
IVS7-45T>C	Canzian, 2008 [50]	404 (52)	369 (48)	513 (51)	496 (49)	0.29	0.94 (0.78-1.14)
IVS7+14delA	Canzian, 2008 [50]	472 (61)	307 (39)	618 (60)	406 (40)	0.23	0.99 (0.82-1.20)
COX-2							
-1329G>A	Liu, 2006 [32]	106 (26)	308 (74)	105 (25)	322 (75)	0.50	0.95 (0.69-1.29)
-899G>C	Liu, 2006 [32]	373 (90)	41 (10)	384 (90)	43 (10)	0.05	0.98 (0.63-1.54)
	Pereira, 2006 [31]	27 (73)	10 (27)	130 (62)	80 (38)	0.22	0.60 (0.28-1.31)
Pooled OR							**0.86 (0.56-1.32)**
IVS5-275T>G	Canzian, 2008 [50]	467 (61)	299 (39)	611 (61)	392 (39)	0.22	1.00 (0.82-1.21)
V102V (G>C)	Canzian, 2008 [50]†	619 (77)	183 (23)	839 (80)	214 (20)	0.11	1.16 (0.93-1.45)
G587R (G>A)	Liu, 2006 [32]	379 (96)	14 (4)	384 (94)	23 (6)	0.03	0.62 (0.31-1.22)
*429T>C	Canzian, 2008 [50]	332 (42)	468 (58)	398 (38)	644 (62)	0.38	0.87 (0.72-1.05)

DH, dominant homozygous; VAC, variant allele carriers; †, contact with authors (through e-mail) was established to recover the discriminated genotypes distribution

Estimated risk of *COX-2* polymorphisms

The – 899G>C was the only *COX-2* polymorphism addressed in more than one study. No heterogeneity between the two studies was observed, although different ethnic populations were studied.[35,36] No modified risk for AIM was noticed for -899C allele carriers. It should be mentioned that three *COX-2* SNPs: G587R, V102V, and *429T>C showed association trends for AIM onset.

Risk of gastric adenocarcinoma

Estimated risk of *COX-1* polymorphisms

The two studies evaluating the L237M and V481I *COX-1* polymorphisms were homogeneous, although reporting two different ethnical populations.[53,55] No statistically significant results were observed.

Table 3. Random-effects *Odds Ratio* (unadjusted) and 95%CI estimated in this analysis following the dominant model of inheritance for gastric cancer onset.

Polymorphism	First author, year [ref]	Cases (%)		Controls (%)		Variant allele frequency	OR (95% CI)
		DH	VAC	DH	VAC		
COX-1							
Q41Q (A>G)	Canzian, 2008 [50]†	107 (95)	6 (5)	957 (92)	84 (8)	0.04	0.64 (0.27-1.50)
G213G (C>A)	Canzian, 2008 [50]	72 (63)	42 (37)	613 (58)	439 (42)	0.24	0.81 (0.55-1.21)
L237M (C>A)	Canzian, 2008 [50]†	112 (97)	3 (3)	1020 (97)	33 (3)	0.02	0.83 (0.25-2.74))
	Hou, 2007 [52]	292 (96)	13 (4)	402 (97)	14 (3)	0.02	1.28 (0.59-2.76)
Pooled OR							**1.13 (0.59-2.15)**
V481I (G>A)	Canzian, 2008 [50]†	113 (97)	3 (3)	1038 (99)	12 (1)	0.01	2.30 (0.64-8.26)
	Hou, 2007 [52]	317 (99)	4 (1)	429 (99)	3 (1)	0.00	1.80 (0.40-8.12)
Pooled OR							**2.08 (0.78-5.50)**
IVS7-45T>C	Canzian, 2008 [50]	58 (52)	53 (48)	513 (51)	496 (49)	0.29	0.95 (0.64-1.40)
IVS7+14delA	Canzian, 2008 [50]	72 (66)	37 (34)	618 (60)	406 (40)	0.23	0.78 (0.52-1.19)
COX-2							
-1423A>G	Zhang, 2006 [33]	283 (88)	40 (12)	592 (92)	54 (8)	0.04	1.55 (1.01-2.39)¦
-1329G>A	Liu, 2006 [32]	73 (19)	316 (81)	105 (25)	322 (75)	0.50	1.41 (1.01-1.98)‡¦
	Zhang, 2006 [33]	32 (10)	291 (90)	136 (21)	510 (79)	0.52	2.43 (1.61-3.66)¦
Pooled OR							**1.83 (1.07-3.10)¦**
-899G>C	Liu, 2006 [32]	346 (89)	42 (11)	384 (90)	43 (10)	0.05	1.08 (0.69-1.70)‡
	Zhang, 2006 [33]	288 (89)	35 (11)	620 (96)	26 (4)	0.02	2.90 (1.71-4.91)
	Pereira, 2006 [31]	36 (49)	37 (51)	130 (62)	80 (38)	0.22	1.67 (0.98-2.86)
	Saxena, 2008 [49]	14 (23)	48 (77)	171 (71)	70 (29)	0.16	8.38 (4.34-16.16)¦
	Hou, 2007 [52]	210 (72)	80 (28)	288 (70)	121 (30)	0.16	0.91 (0.65-1.27)
Pooled OR							**2.02 (1.00-4.10)¦§**
IVS5-275T>G	Canzian, 2008 [50]	72 (67)	36 (33)	611 (61)	392 (39)	0.22	0.78 (0.51-1.19)
	Hou, 2007 [52]	218 (70)	93 (30)	298 (71)	123 (29)	0.16	1.03 (0.75-1.42)
Pooled OR							**0.93 (0.71-1.21)**
V102V (G>C)	Canzian, 2008 [50]†	83 (72)	32 (28)	839 (80)	214 (20)	0.11	1.51 (0.98-2.3)
	Hou, 2007 [52]	230 (76)	72 (24)	285 (70)	125 (30)	0.16	0.71 (0.51-1.00)
Pooled OR							**1.03 (0.49-2.14)§**
G587R (G>A)	Liu, 2006 [32]	368 (95)	18 (15)	384 (94)	23 (6)	0.03	0.82 (0.43-1.54)‡
*429T>C	Canzian, 2008 [50]	53 (49)	56 (51)	398 (38)	644 (62)	0.38	0.65 (0.44-0.97)¦
	Hou, 2007 [52]	137 (45)	167 (55)	165 (40)	251 (60)	0.36	0.80 (0.59-1.08)
Pooled OR							**0.74 (0.59-0.94)¦**
IVS7+111T>C	Hou, 2007 [52]	221 (74)	78 (26)	307 (73)	114 (27)	0.15	0.95 (0.68-1.33)
*2430C>T	Hou, 2007 [52]	289 (94)	19 (6)	399 (98)	10 (2)	0.01	2.62 (1.20-5.73)¦

DH, dominant homozygous; VAC, variant allele carriers; †, contact with authors (through e-mail) was established to recover the discriminated genotypes distribution; ‡, dysplasia and gastric adenocarcinoma pooled together; ¦, studies with statistical significant results (P<0.05); §, heterogeneity detected across studies (P<0.05).

Estimated risk of *COX-2* polymorphisms

Considering only functionally expected polymorphisms (Addenda 2), the analysis of -899G>C revealed heterogeneity across studies that could not be explained even after data stratification by ethnicity. A 2.02-fold impact was observed for -899C allele carriers (95% CI: 1.00–4.10, $P_{heterogeneity}$<0.001). Likewise, the -1329G>A *COX-2* polymorphism conferred

an increased susceptibility of 1.83 (95%CI: 1.07–3.10) in A allele carriers and of 2.18 among AA genotype carriers (95% CI: 1.36–3.49) for GC development. The *429T>C analysis revealed that C allele carriers had a protection for GC onset (OR=0.74; 95%CI: 0.59–0.94).

For the nonfunctional expected V102V polymorphism the two reported studies seemed to be heterogeneous ($P_{heterogeneity}$=0.007), showing strong opposing trends that might be explained as both studies were developed in different ethnical populations. The -1423A>G and *2430C>T noncoding region polymorphisms, addressed only once by Zhang *et al.*[31] and by Hou *et al.*,[55] respectively, revealed an increased risk of GC.

2.3.6. Colorectal carcinogenesis and *COX* polymorphisms

A total of 19 *COX* polymorphisms were considered throughout 16 studies[37–51,54] assessing their impact on colorectal carcinogenesis. Five were identified within the coding region of *COX-1* and 14 throughout *COX-2* gene. The random-effect ORs, under the dominant genetic model, for the onset of colorectal lesions are characterized in Tables 4 and 5. We did not observe any obvious publication bias for either *COX-1* or *COX-2* polymorphisms in colorectal tumors.

Table 4. Random-effects *Odds Ratio* (unadjusted) and 95%CI estimated in this analysis following the dominant model of inheritance for colorectal adenoma onset.

Polymorphism	First author, year [ref]	Cases (%)		Controls (%)		Variant allele frequency	OR (95% CI)
		DH	VAC	DH	VAC		
COX-1							
L15_L16del	Ulrich, 2004 [34]	510 (98)	11 (2)	616 (99)	5 (1)	0.00	2.66 (0.92-7.70)
R8W (C>T)	Siezen, 2006 [37]†	334 (90)	39 (10)	339 (87)	53 (14)	0.07	0.75 (0.48-1.16)
	Ulrich, 2004 [34]	445 (85)	76 (15)	539 (87)	82 (13)	0.07	1.12 (0.80-1.57)
Poole OR							**0.94 (0.63-1.40)**
L237M (C>A)	Siezen, 2006 [37]†	300 (96)	14 (4)	332 (97)	12 (3)	0.02	1.29 (0.59-2.84)
	Ulrich, 2004 [34]	493 (95)	28 (5)	585 (94)	36 (6)	0.03	0.92 (0.56-1.53)
Pooled OR							**1.02 (0.66-1.56)**
P17L	Ulrich, 2004 [34]	451 (87)	70 (13)	527 (85)	94 (15)	0.08	0.87 (0.62-1.22)
COX-2							
-1462_-1461delTG	Ali, 2005 [36]	702 (96)	32 (4)	704 (94)	45 (6)	0.03	0.71 (0.45-1.14)
-1329G>A	Siezen, 2006 [37]	22 (6)	349 (94)	16 (4)	377 (96)	0.80	0.67 (0.35-1.30)
	Ueda, 2008 [51]	106 (23)	349 (77)	227 (22)	725 (78)	0.53	0.91 (0.70-1.18)
Pooled OR							**0.87 (0.68-1.11)**
-899G>C	Siezen, 2006 [37]†‡	237 (70)	100 (30)	274 (74)	94 (26)	0.13	1.23 (0.88-1.71)
	Gunter, 2006 [38]	151 (72)	59 (28)	141 (72)	55 (28)	0.15	1.00 (0.65-1.54)
	Ulrich, 2005 [35]	344 (70)	150 (30)	405 (69)	179 (31)	0.17	0.99 (0.76-1.28)
	Ueda, 2008 [51]	440 (97)	15 (3)	989 (94)	62 (6)	0.03	0.54 (0.31-0.97)¦
Pooled OR							**0.96 (0.74-1.25)**
-798A>G	Ali, 2005 [36]	490 (66)	251 (34)	493 (66)	257 (34)	0.19	0.98 (0.79-1.22)
IVS5-275T>G	Ali, 2005 [36]	534 (71)	213 (29)	530 (70)	223 (30)	0.17	0.95 (0.76-1.18)
	Gunter, 2006 [38]	149 (71)	61 (29)	136 (69)	61 (31)	0.16	0.91 (0.60-1.40)
Pooled OR							**0.94 (0.77-1.15)**
V102V (G>C)	Siezen, 2006 [37]†	284 (74)	100 (26)	267 (66)	136 (34)	0.19	0.69 (0.51-0.94)¦
	Gunter, 2006 [38]	154 (73)	56 (27)	142 (72)	55 (28)	0.15	0.94 (0.61-1.45)
Pooled OR							**0.77 (0.58-1.03)**
V511A (T>C)	Lin, 2002 [39]† USS/K study	113 (95)	6 (5)	109 (90)	12 (10)	0.05	0.48 (0.17-1.33)
	Lin, 2002 [39]† UNC study	39 (93)	3 (7)	88 (90)	10 (10)	0.05	0.68 (0.18-2.60)
Pooled OR							**0.55 (0.24-1.23)**
*429A>G	Siezen, 2006 [37]	159 (42)	219 (58)	196 (50)	200 (50)	0.30	1.35 (1.02-1.79)
	Gunter, 2006 [37]	30 (14)	180 (86)	18 (9)	179 (91)	0.65	0.60 (0.32-1.12)
	Ali, 2005 [36]	311 (41)	438 (58)	347 (46)	409 (54)	0.33	1.19 (0.97-1.47)
Pooled OR							**1.11 (0.82-1.51)**
*1806A>G	Ueda, 2008 [51]	451 (99)	4 (1)	1042 (99)	9 (1)	0.00	1.03 (0.31-3.35)

DM, dominant homozygous; VAC, variant allele carriers; †, contact with authors (through e-mail) was established to recover the discriminated genotypes distribution**;** ‡, genotype distributions given by the author. These frequencies do not overlap with the ones presented in the original paper; UNC, University of North Carolina study; USC/K, University of Southern California / Kaiser study; ¦, studies with statistical significant results (P<0.05).

Risk of colorectal adenoma

Estimated risk of *COX-1* polymorphisms

The studies by Ulrich *et al.*[37] and Siezen *et al.*[40] addressed, altogether, four COX1 polymorphisms (L15_L16del, R8W, P17L and L237M) in colorectal adenoma. For the

functional expected polymorphisms, R8W, P17L, and L237M polymorphisms,[68–71] no genetic effect was observed. The L15_L16del *COX-1* polymorphism, although characterized only once by Ulrich *et al.*,[37] it seemed to increase the risk of colorectal adenoma, although not reaching the significance level.

Estimated risk of *COX-2* polymorphisms

Nine *COX-2* polymorphisms (-1462_-1461delTG, -1329G>A, -899G>C, -798A>G, IVS5-275T>G, V102V, V511A, *429T>C, and *1806A>G) were appraised for colorectal adenoma in a total of six studies.[38–42,54] Only the pooled data for the V102V and V511A suggested a protective trend for colorectal adenoma. Excluding the study by Gunter *et al.*[41] from the pooled analysis of *429T>C *COX-2* polymorphism, (C allele frequency very different from all the others in the control populations) we detected an OR of 1.25 (95% CI: 1.06–1.47) in C allele carriers. No influence on adenoma onset susceptibility was noticed for the -899G>C and -1329G>A *COX-2* polymorphisms. The sensibility was tested by omitting the study by Ali *et al.*[39] from the IVS5 -275G>T analysis. No change in the SNP behavior was observed.

Risk of colorectal cancer

Estimated risk of *COX-1* polymorphisms

Three studies characterized *COX-1* polymorphisms.[46–48] No gene–disease association was noticed for both R8W and V481I *COX-1* coding region SNPs. Contradictory data were presented in the individual studies assessing L237M SNP in Caucasians.[47,48] Owing to the limited number of studies we were not able to identify the source of heterogeneity ($P_{\text{heterogeneity}}$=0.006).

Table 5. Random-effects *Odds Ratio* (unadjusted) and 95%CI estimated in this analysis following the dominant model of inheritance for colorectal cancer onset.

Polymorphism	First author, year [ref]	Cases (%)		Controls (%)		Variant allele frequency	OR (95%CI)
		DH	VAC	DH	VAC		
COX-1							
R8W (C>T)	Siezen, 2006b [45]†	168 (84)	33 (16)	335 (86)	56 (14)	0.08	1.18 (0.74-1.88)
L237M (C>A)	Siezen, 2006b [45]†	182 (96)	8 (4)	348 (92)	31 (8)	0.04	0.49 (0.22-1.10)
	Goodman, 2004 [44] (Caucasians)	161 (90)	17 (10)	309 (96)	14 (4)	0.02	2.33 (1.12-4.85)
	Goodman, 2004 [44] (African-Americans)	113 (99)	1 (1)	184 (96)	7 (4)	0.02	0.23 (0.03-1.92)
Pooled OR (Caucasians)							**1.08 (0.23-4.98)**
V481I (G>A)	Landi, 2006 [43]	280 (99)	3 (1)	265 (98)	5 (2)	0.01	0.57 (0.13-2.40)
	Goodman, 2004 [44] (Caucasians)	173 (98)	3 (2)	324 (98)	6 (2)	0.01	0.94 (0.23-3.79)
	Goodman, 2004 [44] (African-Americans)	115 (100)	0 (0)	196 (99)	1 (1)	0.00	-
Pooled OR (Caucasians)							**0.73 (0.27-2.00)**
COX-2							
-1423A>G	Cox, 2004 [42]	201 (73)	76 (27)	181 (70)	78 (30)	0.17	0.88 (0.60-1.28)
	Tan, 2007 [41]	914 (91)	86 (9)	1180 (91)	120 (9)	0.05	0.93 (0.69-1.24)
Pooled OR							**0.91 (0.72-1.14)**
-1329G>A	Siezen, 2006b [45]	10 (5)	186 (95)	20 (5)	371 (95)	0.79	1.00 (0.46-2.19)
	Tan, 2008 [41]	178 (18)	822 (82)	300 (23)	1000 (77)	0.50	1.39 (1.13-1.70)‡
Pooled OR							**1.36 (1.11-1.66)‡**
-899G>C	Cox, 2004 [42]	150 (68)	70 (32)	170 (66)	87 (34)	0.19	0.91 (0.62-1.34)
	Koh, 2004 [46]†	273 (88)	37 (12)	1067 (91)	110 (9)	-	1.31 (0.89-1.95)
	Hamajima, 2001 [40]	140 (95)	8 (5)	230 (95)	11 (5)	0.02	1.19 (0.47-3.04)
	Tan, 2007 [41]	919 (92)	81 (8)	1237 (95)	63 (5)	0.02	1.73 (1.23-2.43)‡
	Xing, 2008 [48]	119 (87)	18 (13)	169 (85)	30 (15)	0.08	0.85 (0.45-1.60)
Pooled OR							**1.21 (0.90-1.61)**
Pooled OR (Asians)							**1.35 (1.01-1.81)‡**
-646C>T	Goodman, 2004 [44]† (Caucasians)	175 (99)	1 (1)	331 (100)	1 (0)	0.00	1.89 (0.12-30.42)
	Goodman, 2004 [44]† (African-Americans)	104 (91)	10 (9)	181 (91)	17 (9)	0.04	1.02 (0.45-2.32)
Pooled OR							**1.08 (0.49-2.36)**
-196C>G	Cox, 2004 [42]	280 (97)	10 (3)	263 (98)	6 (2)	-	1.57 (0.56-4.37)
-125T>G	Hamajima, 2001 [40]	141 (97)	5 (3)	230 (97)	7 (3)	0.01	1.17 (0.36-3.74)
IVS5-275T>G	Cox, 2004 [42]	187 (64)	103 (36)	174 (64)	97 (36)	0.20	0.99 (0.70-1.40)
V102V (G>C)	Cox, 2004 [42]	180 (62)	110 (38)	183 (67)	89 (33)	0.18	1.26 (0.89-1.78)
	Siezen, 2006b [45]	142 (70)	61 (30)	287 (72)	111 (28)	0.15	1.11 (0.77-1.61)
Pooled OR							**1.19 (0.92-1.53)**
V511A (T>C)	Goodman, 2004 [44]† (Caucasians)	177 (100)	0 (0)	329 (100)	0 (0)	0.00	-
	Goodman, 2004 [44]† (African-Americans)	109 (95)	6 (5)	186 (93)	14 (7)	0.04	0.73 (0.27-1.96)
	Lin, 2002 [39]†	129 (93)	9 (7)	237 (92)	21 (8)	0.04	0.79 (0.35-1.77)
	Sansbury, 2006 [47]	223 (93)	17 (7)	292 (90)	34 (10)	0.05	0.65 (0.36-1.20)
Pooled OR							**0.71 (0.46-1.09)**
*429A>G	Cox, 2004 [42]	140 (48)	150 (52)	126 (47)	145 (53)	0.31	0.93 (0.67-1.30)
	Siezen, 2006b [45]	97 (48)	103 (52)	190 (49)	198 (51)	0.30	1.02 (0.72-1.43)
Pooled OR							**0.97 (0.77-1.23)**
*1806A>G	Cox, 2004 [42]	257 (91)	24 (9)	258 (96)	10 (4)	-	2.41 (1.13-5.14)‡
	Siezen, 2006b [45]†	194 (97)	5 (3)	368 (95)	21 (5)	0.03	0.45 (0.17-1.22)
Pooled OR							**1.08 (0.21-5.56)¦**
*2291G>A	Cox, 2004 [42]	245 (92)	20 (8)	241 (96)	10 (4)	-	1.97 (0.90-4.29)

DH, dominant homozygous; VAC, variant allele carriers; †, contact with authors (through e-mail) was established to recover the discriminated genotypes distribution; ‡, studies with statistical significant results (P<0.05); ¦, heterogeneity detected across studies (P<0.05).

Estimated risk of *COX-2* polymorphisms

Nine studies were conducted in Caucasian, African–American, or Asiatic populations, gathering a total of 12 *COX-2* polymorphisms examined.[42–45,47–51] The overall random-effect OR for -899G>C polymorphism was 1.21 (95% CI: 0.90–1.6). This value achieved statistical significance in Asiatic populations (OR=1.35; 95% CI: 1.01–1.81).[43,44,49,51] A 1.36-fold increased risk of CRC development was also observed for the -1329G>A polymorphism (95% CI: 1.11–1.66) (see Figure 2).

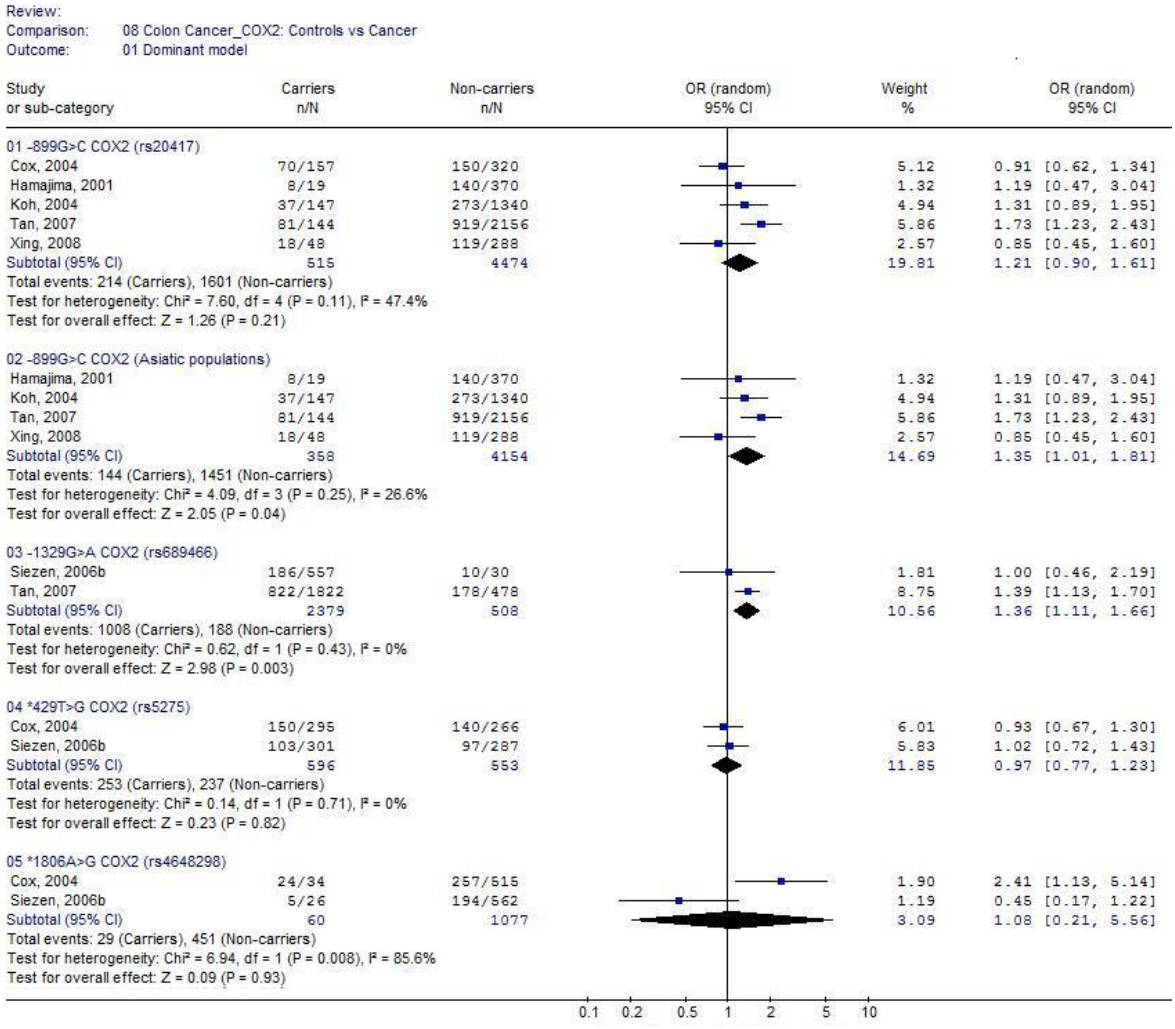

Figure 2. Forest plot describing the random effect ORs and 95% CI from studies assessing the association between *COX-2* polymorphisms and colorectal cancer: (01) role of – 899G>C polymorphism in colorectal cancer (CRC) onset; (02) role of – 899G>C polymorphism in CRC only in Asiatic populations; (03) role of -1329G>A polymorphism at CRC onset; (04) role of *429T>C polymorphism in CRC development and (05) role of *1860A>G polymorphisms in CRC onset.. All analysis followed the dominant genetic model. I^2 and *P* value for ^{2} of heterogeneity is reported for each group analysis. CI, confidence interval; OR, odds ratio.

The *429T>C polymorphism which was associated with increased risk of colorectal adenoma, did not seem to play any role in the development of CRC. Strong heterogeneity was detected across the two studies[45,48] enrolled involving *1806A>G polymorphism ($P_{heterogeneity}$=0.004). Several study characteristics could be used to explain this lack of homogeneity, but the limited number of studies restricted the interpretation of this heterogeneity. The V511A *COX-2* polymorphism, only identified in African–Americans, showed a protective tendency for CRC. All the other genetic variations did not alter the susceptibility to developed CRC, although the *2291G>A SNP, assessed once by Cox *et al.*,[45] showed a strong increased risk trend for cancer.

No single study addressed the CRC risk onset in colorectal adenoma patients.

2.4. DISCUSSION

In November 2002, Lin *et al.*[42] reported the first study addressing *COX* polymorphisms in the susceptibility for CRC onset. Nearly 6 years later, more than 20 studies were developed gathering a total of 26 *COX* polymorphisms appraised in gastrointestinal tumors: nine in *COX-1* and 17 in *COX-2* genes.[31,35–55] Now, do we have conclusive results that could lead to clinical reasoning or research?

2.4.1. Role of *COX* polymorphisms in gastric carcinogenesis

The different roles that COX-1 and COX-2 enzymes portray, COX-1 associated with housekeeping functions and COX-2 with inflammation and tumor development,[19] may explain the effort disparity to assess *COX-1* or *COX-2* polymorphisms in gastric and colorectal tumors. COX-1 enzyme in stomach is involved in the protection and maintenance of gastric mucosa;[19] therefore it would be interesting to appraise the involvement of *COX-1* polymorphisms in the development of gastric lesions, since it was only assessed in two studies.[53,55]

In gastric carcinogenesis, polymorphisms in *COX-2* gene seemed to differently influence the genetic susceptibility according to the type of gastric lesions assessed (AIM or GC). Despite the publishing of four papers since last year,[52,53,55,57] the restricted number of studies included in this analysis (n=6)[31,35,36,52,53,55] only allowed us to draw some remarks and not strong conclusions. Two of the most studied polymorphisms in the promoter region of *COX-2* gene (-1329G>A and -899G>C) seemed to be associated with susceptibility for gastric cancer onset. The -1329A allele was associated with a 1.83-fold increased risk of gastric cancer in approximately 1800 Asiatic individuals. In the study carried out by Liu *et al.*[36] the increased susceptibility was even higher in individual carriers of -1329AA genotype positives for *H. pylori* infection (OR=3.88; 95% CI: 1.46–10.34) or smokers (OR=7.02; 95% CI: 2.19–22.48). Furthermore, although not addressed in the original study, this polymorphism also seemed to be involved in the development of gastric cancer in patients with intestinal metaplasia (OR=1.49; 95% CI: 1.06–2.09). All these associations can be biologically supported, because the -1329A allele creates a core recognition sequence for the c-MYB nuclear transcription factor resulting in higher transcription activity of *COX-2* as it was proved in the study by Zhang *et al.*[27] Further studies focusing different ethnical population are important to understand whether this polymorphism behavior is ethnic-specific, as the only study addressed in adult Caucasians[48] did not show any impact on gastrointestinal cancer onset. These should also address several confounding factors like age, gender, H. pylori infection, and smoking status.

An increased risk behavior for the development of GC was observed or the -899G>C polymorphism (OR=2.02; 95% CI: 1.00–4.10), although strong heterogeneity was reported that could not be interpreted even after ethnicity and type of controls stratification. From the study by Liu *et al.*[36] we defined individuals with superficial gastritis and chronic atrophic gastritis as belonging to the non-lesions control group. This may not be the best approach since these lesions might already have some resemblances with malignant tumors, thus explaining the non-association result observed in this individual study. Another study

worth mentioning is the one by Saxena *et al.*[52] where we observed a nine-fold increased risk of GC in a Northern Indian population. This value was significantly higher than all other ethnical populations addressed, suggesting that the contribution of genetic polymorphims may be dependent on the population being studied, as well as on several environmental and dietary factors that influence that population.[72] The molecular mechanism and the biological impact of this polymorphism in cancer development are surrounded by controversy. First characterized by Papafili *et al.*[73] is recognized as a functional polymorphism since the transversion from a guanine (G) to a cytosine (C) in the promoter region of *COX-2* gene might inhibit the binding of the Sp1 positive transcription factor resulting in a reduction of the promoter activity. In contrast, Szczeklik *et al.*[74] reported that monocytes from – 899CC genotype carriers had a 10-fold increase in the production of PG. In addition, Zhang *et al.*[27] also observed that heterozygous individuals seemed to have a higher COX-2 mRNA expression although not being statistically significant. The latter results could be explained as the -899C allele, besides eliminating a Sp1 recognition binding site, also creates and E2F homology binding region, based on bioinformatic programmes, that could lead to a higher transcription activity.[27,74] All of these findings suggest that different cell types under different physiological conditions could determine the -899G>C behavior by the binding of specific nuclear proteins to the promoter region.[31] Therefore, further functional studies in gastric tumors are required to elucidate the molecular mechanism involving the -899C allele in gastric carcinogenesis. Unlike most of the SNPs addressed, the *429T>C SNP seemed to play a protective role in both AIM and GC development. Although these findings warrant further studies, future investigations should focus on the combined influence, haplotype analysis of the -1329G>A, -899G>C, and *429T>C COX-2 polymorphisms. Ultimately, this could allow the identification of higher-risk individuals for gastric cancer development that may beneficiate from chemopreventive and/or follow-up strategies.

2.4.2. Role of *COX* polymorphisms in colorectal carcinogenesis

We can safely say that CRC is one of the most studied models for unraveling the role of COX enzymes in several key steps of carcinogenesis.[75] Nowadays, it is well established that COX-2 plays a pivotal role in early colorectal carcinogenesis and that the use of NSAIDs, like aspirin, is associated with decreased risk for adenoma and CRC onset and recurrence.[76–79]

All the polymorphisms in *COX-1* gene were investigated in over 1000 individuals. Only the L15_L16del had any impact on the genetic susceptibility presenting a strong increased risk trend for colorectal adenoma onset in approximately 1200 Caucasians in a hospital-based study carried out by Ulrich *et al.*[37] Curiously, this increased susceptibility is even more evident in non-regular users (OR=8.58; 95% CI: 1.07–68.94) and annulled in regular users of NSAIDs (OR=1.08; 95% CI: 0.24–4.87), confirming that at least part of the anti-inflammatory tumor protective effects of NSAIDs are because of COX inhibition.[16] The remaining polymorphisms expected to have an impact on COX-1 function, following the sequence homology-based software programmes predictions,[68,69] did not show any association. An interesting heterogeneity was detected among the two Caucasian studies addressing the L237M polymorphism[47,48] revealing opposing estimates. Owing to the restricted number of studies we did not have the capacity to scrutinize the characteristic that could explain this heterogeneity, as they diverge in several features (demographic origin, study design, number of participants). We will have to wait for further studies to unravel the true meaning of this SNP in CRC development.

COX-2 is the inducible isoform of COX enzymes.[19] Unlike COX-1 that is constitutively expressed in most tissues, COX-2 expression is mainly regulated at transcription level, although posttranscriptional level regulation (COX-2 mRNA stability) also seems to influence COX-2 expression.[80,81] *COX-2* promoter region has several recognition sites for nuclear proteins, including Sp1, c-MYB, NF-kB, AP1, TATA.[80] Therefore, it is not surprising that eight of the fourteen *COX-2* polymorphisms analyzed for colorectal lesions

onset belong to the promoter region (-1462_-1461delTG, -1423A>G, -1329G>A, -899G>C, -798A>G, -646C>T, -196C>G, -125T>G), one to the intronic region (IVS5 – 275T>G), two to the coding regions (V102V and V511A) and three to the 3' untranslated region (*429T>C, *1806A>G, *2291G>A).

Seven *COX-2* polymorphisms, -1329G>A, -899G>C, IVS5-275T>G, V102V, V511A, *429T>C and *1806A>G, were investigated in both adenoma and CRC susceptibility, and all of them, except for the IVS5-275T>G and V511A, seemed to have a histological type-dependent behavior. The V511A polymorphism, identified only in African-Americans, revealed a non-significant histological-type independent protection for colorectal tumors. This lack of association was possibly due to the low frequency of 511A variant (5%). The in vitro functional characterization of this polymorphism, that leads to an amino acid change close to COX-2 active site, did not revealed any allele differences in the enzyme kinetic parameters ($V_{máx}$ and Km) or stability for arachidonic acid utilization.[42] Nevertheless, this absence of functional differences between alleles could be limited by conditions in vitro, as mentioned by Lin *et al.*[42]

The pooled analysis for the -899G>C *COX-2* polymorphism revealed an increased risk association for CRC development in Asiatic adults, although this was not noticed in all individual studies.[43,51] Nevertheless, these studies had a low number of participants (~350) and as the frequency of this polymorphism is very low in Asiatic populations (2 to 8%), the lack of association may represent low statistical power. Likewise, the -1329G>A genetic variation also revealed an increased risk of CRC, although it was considered only in the study by Tan *et al.*[44] which gathered a total of 2300 Asiatic individuals following a population-based design ensuring a statistically significant result. In Caucasians, we did not have enough statistical power to detect any association for either polymorphism. Both polymorphims seemed to have the same increased risk behavior, independent of the tumor locations, but only associated with the more severe forms of gastrointestinal lesions (GC and CRC). In contrast, the *429T>C and V102V *COX-2* polymorphisms seemed to

influence the development of colorectal lesions in early stages of carcinogenesis. The *429T>C SNP, associated with protection for GC development, in colorectal lesions exposed a 1.25-fold increased risk of adenoma onset in Caucasians, suggesting an organ-specific involvement. The thymine (T) to cytosine (C) exchange in an AU-rich elements region, known to influence mRNA degradation,[81] could enhance the stability of mRNA transcripts that could ultimately lead to an increased PG production.[39,82] This biologic assumption seems to support our outcome in colon, but as, so far, no study has functionally characterized this SNP we can only wait for future evidences. Such a clear association for adenoma development was not detected but instead a protective trend was noticed with the synonymous V102V polymorphism. Further and larger studies are necessary to elucidate this relationship. The low-frequency *1806G allele identified in the 3' untranslated region of exon 10 is believed to have an impact on mRNA COX-2 stability through the addition of some poly-A tail to the mRNA, generating a longer and more stable mRNA.[45] Conflicting results were observed between the two individual studies[45,48] carried out with this polymorphism. Owing to the limited number of studies we were not able to identify the source of heterogeneity, although they only seemed to diverge in one feature. The study by Cox *et al.*[45] had a hospital-based design and in contrast the study by Siezen *et al.*[48] followed a population-based one. Before any remarks could be drawn, further larger epidemiological studies, also as functional tests, are recommended to elucidate the nature of *1806G allele in CRC.

A systematic review can be a resourceful tool in detecting an association that could otherwise remain masked in studies with a small number of participants,[83] especially in those evaluating rare allele frequency polymorphisms. Nevertheless, these results should be interpreted bearing in mind the limitations encountered in this analysis. Firstly, the elevated number of *COX* polymorphisms addressed and the lack of genotype frequency information for each one in each of the studies did not allow the estimation of the best genetic model of inheritance to follow. Therefore, we assumed the dominant model for every SNP. For

some polymorphisms this model might not be the most suitable to allow a clear assessment of the gene-disease interaction.[83] Secondly, for most polymorphisms we were not able to address the sources of heterogeneity when detected among studies, and to perform subgroup stratifications analysis, because of the limited number of published studies. Thirdly, in at least one study there was a clear age gap between cases and controls. The control group was younger than the patients, meaning that possibly there are participants that could develop cancer before reaching the median age of cases in the control group. Fourthly, several studies did not confirm the absence of lesions in the control group by any technique and several others did not mention the method of choice. The last two drawbacks could ultimately lead to an underestimation of the overall polymorphism effect. Finally, this systematic review was based on unadjusted data, as the genotype information stratified for the main confounding variables was not available in the original papers and also the confounding factors addressed across the different studies were variable. The adjusted estimates could give more precise and strong associations, as they reduce the impact of possible confounding factors.

In future studies several requirements should be fulfilled, concerning not only the study design but also the reporting of data: a sample size estimation based on the genotype distribution should be carried out, especially for low-frequency alleles; the inclusion and exclusion criteria ought to be stated clearly in the reporting manuscript; the control group should represent the same source populations as cases and the main characteristics, age, and gender, should be matched between the two groups; to ensure the correct classification, all participants should be screened for possible gastric or colorectal lesions; this information alongside the validated technique applied should be facilitated when reporting the study; the selection of participants as well as the genotyping examination should be performed by blind personnel; and finally, potential confounders like ethnicity, *H. pylori* (for gastric lesions), diet, NSAID use and lifestyle habits should be managed by subgroup analysis. We may conclude that, although further research is needed, there are

apparently consistent results (Table 6), both laboratory and observational, in *COX-2* polymorphisms that may help to select a group of patients at higher risk of gastric cancer (-899G>C, -1329G>A, and *429T>C).

Table 6. *COX* polymorphisms expected impact based on bioinformatics predictive programs /functional studies (rows) and observed estimates (OR) on current study (columns)

***COX* polymorphisms**	**Gastric Carcinogenesis**		**Colorectal Carcinogenesis**	
	AIM	**Gastric Cancer**	**Colorectal Adenoma**	**Colorectal Cancer**
Funcional				
COX-1				
R8W			↔	↔
P17L			↔	
L237M	↔	↔	↔	§
COX-2				
-1423A>G		↑↑↑		↔
-1329G>A	↔	↑↑↑	↔	↑↑↑
-899G>C	↔	↑↑↑	↔	↑↑↑†
IVS5-275T>G	↔	↔	↔	↔
*429T>C	↓↓	↓↓↓	↑↑↑¦	↔
*1806A>G			↔	§
Non-functional				
COX-2				
-1462_-1461delTG			↓	
-798A>G			↔	
V102V	↑	§	↓↓	↑
V511A			↓‡	↓↓‡
Unknown				
COX-1				
IVS7+14delA	↔	↔		
IVS7-45T>C	↔	↔		
L15-L16del			↑↑	
V481I	↔	↑		↔
Q41Q	↔	↔		
G213G	↔	↔		
COX-2				
-646C>T				↔
-196C>G				↔
-125T>G				↔
IVS7+111T>C		↔		
G587R	↓	↔		
*2291G>A				↑↑
*2430C>T		↑↑↑		

AIM, atrophy or/and intestinal metaplasia; ↔, no association; ↓, trend, ↓↓, strong trend, ↓↓↓, statistical association (P<0.05) for protection. Protection is define as the 95%CI for OR<1; ↑, trend, ↑↑, strong trend, ↑↑↑, statistical association (P<0.05) for risk. Risk is define as the 95%CI for OR>1; §, heterogeneity detected among studies; ¦, excluding the study by Gunter *et al.*[38] that has a C allele distribution very different from the other populations; †, in Asiatic populations; ‡, in African-American individuals.

Furthermore, in sporadic colon adenoma (V102V, V511A and *429T>G) and cancer (-1329G>A, -899G>C, V511A) *COX-2* polymorphisms may help in defining a genetic profile of risk. If confirmed in future studies such as cohort studies or else (e.g., cost-effectiveness analysis), these genetic profiles may enable clinicians to select individuals for early diagnosis strategies, diverse management schedules such as the follow-up of patients with intestinal metaplasia in the stomach or patients with earlier colonic adenoma (by

anticipating follow-up examinations), or even to propose selective COX-2 inhibitors or nonspecific COX inhibitors in patients with precancerous lesions.

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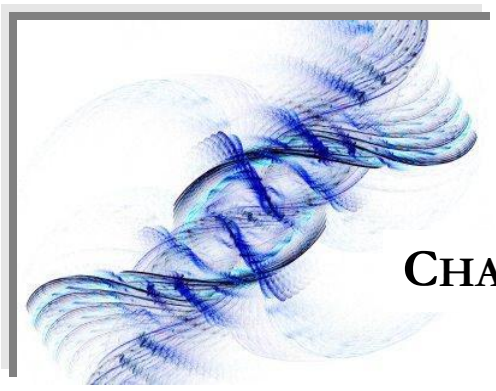

CHAPTER III

ROLE OF *COX-2* POLYMORPHISMS IN COLORECTAL CANCER IN A NORTHERN PORTUGUESE POPULATION

3.1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide, with estimated 1.023.256 new cases and over 529.000 cancer-related deaths every year.[1] In developed countries this scenario worsens, with the CRC being the second most widespread malignancy with a lifetime risk of 5%, mainly imputed to the "westernized lifestyle".[1-3]

The natural evolution of CRC involves the progression of colorectal adenomas to adenocarcinoma, known as the "adenoma-carcinoma sequence", which spans for over 10-15 years thereby providing an undoubtly opportunity for CRC prevention either through early screening or even chemopreventive strategies.[4]

Over the last 150 years, since the originally discovery of Rudolph Virchow, the link between chronic inflammation and tumor development grows stronger, with particularly interest in the colorectal carcinogenesis.[5, 6] The most compelling evidences supporting this inflammation-cancer connection arose from observational and randomized studies that shown a substantial risk reduction (40-50%) in regular users of nonsteroidal anti-inflammatory drugs (NSAIDs).[7-9] In fact, as reviewed by Flossmann and Rothwell,[10] the long-term use of aspirin daily consistently seemed to be effective in the primary prevention of CRC with a latency period of about 10 years.

Traditional NSAIDs such as aspirin are thought to exert their chemopreventive actions mainly by targeting cyclooxygenase (COX) enzymes.[11] There are at least two COXs isoenzymes identified that catalyze the biosynthesis of prostaglandins (PG) and related prostanoids from arachidonic acid.[12] COX-1 is constitutively expressed in a wide range of organs and responsible for normal tissue homeostasis. Whereas, normally undetectable in physiological conditions, COX-2 is induced under inflammatory and tumor promotion stimuli, consequently, increasing the pro-inflammatory PGE_2 tissue levels which are implicated in various carcinogenic pathways by stimulating cell proliferation, angiogenesis,

invasion and inhibiting apoptosis.[12, 13] Moreover, COX-2 overexpression was detected in approximately 50% of adenomas and 85% of colon adenocarcinomas.[14]

In an original observational study that assessed the regular use of aspirin in CRC prevention and its correlation with COX-2 expression, Chan *et al.*[15] observed that aspirin preventive role was only effective in the subgroup of colon cancers overexpressing COX-2 (40% risk reduction) in a dose and treatment duration dependent manner. These results should help decrease the risk/benefits ratio into a more cost-effective approach for the use of COX inhibitors in CRC chemoprevention that is currently hampered by the adverse gastrointestinal (GI) side effects (COX-1 inhibition),[16] by defining a subgroup of individuals that are more likely to beneficiate from NSAIDs protection. Hence, the future challenge lies in the identification of individuals who will express higher levels of COX-2, probably through the interaction between the genetic background and environmental exposure.[17]

As reviewed in a recent paper there are some observational and functional data supporting the involvement of *COX-2* genetic variations in colorectal tumors development. Still, at this point insufficient to support a clinical reasoning.[18] All three polymorphisms addressed in this study were selected on the basis of expected functional relevance or previous associations with colorectal tumors development and on a variant allele frequency in Caucasians higher than 5%. The -1329A>G (rs689466) and -899G>C (rs20417) genetic variations, identified in gene's promoter region that contains several *cis-actin*g regulatory elements, are expected to modulate COX-2 expression by altering the recognition binding site for specific nuclear proteins, thus influencing the genetic susceptibility for CRC onset.[19, 20] On the other hand, the *429T>C (rs5275) polymorphism in an AU-rich elements region (3'UTR) might contribute to cancer development by influencing *COX-2* mRNA stability.[21] In the present study, we sought to evaluate the influence of *COX-2* functional polymorphisms in the development of CRC. We also aimed to investigate

possible interactions between these genetic variations and environmental factors in CRC onset.

3.2. MATERIALS AND METHODS

3.2.1. Study population

This hospital-based case-control study included 373 participants: 117 histologically confirmed CRC patients and 256 cancer-free controls from the northern region of Portugal recruited at the Portuguese Institute of Oncology, Porto. Eligible cases included all patients with a newly diagnosed CRC between January 2002 and September 2007, aged 50 to 75 years, without previous history of inflammatory bowel diseases (IBD) or hereditary syndromes and whom were scheduled for a follow-up observation at our institution between March and May 2008 (n=384). Controls were healthy individuals without any clinical evidence of CRC selected from a DNA database of over one-thousand blood donors that attended our institute between July 2005 and October 2007. All the individuals with age comprised between 50 and 75 years old were included (n=307).

Overall, the participation rate for the approached individuals meeting the cases' inclusion criteria (n=166) was 90% (n=150). We were unable to obtain blood samples from all the included cases by the frame time of this study, but the distribution of known risk factors for CRC (age, gender, body mass index (BMI) and smoking behaviour) did not deviate between the genotyped group and the included population. In the control group, DNA samples were only available from 256 (83%) participants to allow the genotype characterization of all *COX-2* polymorphisms.

Written informed consent was obtained from all subjects prior to their inclusion in the study, according to the Declaration of Helsinki (Addenda 3). Furthermore, this research was approved by the Ethical Committee of the Portuguese Institute of Oncology, Porto.

3.2.2 Data collection and variables definition

At time of recruitment cases were personally interviewed and were asked to recall their lifestyle habits (smoking behavior, BMI) in the year previous to their CRC diagnosis using a questionnaire that was also used to ascertain the demographic characteristics (date of birth, gender, race) and other known risk factors (previous IBD or hereditary syndromes) (Addenda 4). Medical records were reviewed to extract the clinicopathological variables (stage, tumor grade and the presence of synchronous lesions). For the control group the demographic and lifestyle characteristics were retrieved through the Blood donors' bank database search. The age was addressed as a categorical variable defined by overall median age (≥57 years). BMI was defined as the weight divided by the square of the height (kg/m^2) and categorized considering the WHO[22] cut-off defined for overweight people ($\geq 25kg/m^2$). Participants with information about smoking status were stratified as never-smokers and ever-smokers (current and former-smokers).Whenever data available the lifetime exposure to cigarette smoking was computed as cigarette-pack-years (media number of cigarette-packs-day x year).

3.2.3. Sample DNA extraction

Blood samples were collected with a standard venipuncture technique using EDTA containing tubes. Genomic DNA was extracted from peripheral blood leukocytes, using the QIAamp® DNA Blood Mini Kit (Qiagen, Madrid, Spain) following the manufacturer's instructions (Addenda 5).

3.2.4. *COX-2* polymorphisms genotyping

PCR – based restriction fragment length polymorphism (PCR-RFLP) technique was used to characterize the different genotypes of -1329A>G and -899G>C *COX-2* polymorphisms considering the protocols previously described by Zhang *et al.*[20] and Pereira *et al.*[23] The PCR primers sequence, also as other specific genotyping characteristics to each *COX-2* polymorphisms are displayed in Table 1.

Table 1. Primers sequence, annealing temperature, restriction enzymes and digestion products specific for each *COX-2* polymorphisms genotyped through PCR-RFLP

COX-2 Polymorphisms (rs number†)	Primer-pairs	Annealing temperature	Restriction enzyme	RFLP products
-1329A>G (rs689466)				
	F 5'-CCCTGAGACACTACCCATGAT-3' R 5'-GCCCTTCATAGGAGATACTGG-3'	59ºC	*PvuII*	**AA:** 273bp **GA:** 273+220+53bp **GG:** 220+53bp
-899G>C (rs20417)				
	F 5´-ATTCTGGCCATCGCCGCTTC-3' R 5'-CTCCTTGTTTCTTGGAAAGAGACG-3'	60ºC	*Bsh1236I*	**GG:** 134+23bp **GC:** 157+134+23bp **CC:** 157bp

PCR-RFLP, polymerase chain reaction based-restriction fragment length polymorphism; †, single nucleotide polymorphisms identification number in NCBI SNP database; bp, base pairs.

PCRs were performed in a 50μL reaction mixture containing 100ng of DNA that was added to: 0.5 mM of each primer (Metabion, Martinsried, Deutschland), 0.2 mM of each deoxyribonucleotide triphosphate (dNTP) (Fermentas, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 1X Taq buffer and 1U of Taq DNA polymerase (Promega, Madison, WI). The amplification conditions were 95ºC during 5 minutes for the initial denaturation step, followed by 35 cycles of denaturation at 94ºC (1 m), annealing at 60-59ºC (1 m), and extension at 72ºC (1 m). The final extension step consisted of 7 minutes at 72ºC. Afterwards, the amplified fragments were digested with 1U of specific restriction endonucleases (see Table 1) (Fermentas, Vilnius, Lithuania) during 4h at 37ºC and the products visualized in a 2% or 3% (w/v) agarose gel stained with ethidium bromide, allowing the genotype characterization of -1329A>G and -899G>C *COX-2* polymorphisms, respectively. For quality control, (1) negative controls were included in every PCR reaction; (2) the genotype interpretation was independently performed by two experienced researchers. There was no discrepancy between results; (3) a second PCR-RFLP analysis was randomly repeated in ten per cent of all samples for each polymorphism.

The *429T>C polymorphism was genotyped through an allelic discrimination analysis on a ABI Prism® 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following validated TaqMan® SNP genotyping assay (Applied Biosystems, Foster

City, CA, USA): C___7550203_10. The reaction mixture was set up in 6 μL reaction with 50ng of genomic DNA, 2.5 μL of the 2X TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA) and 2.25 μL TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). Cycling was 95ºC for 10 min followed by 40 cycles that consisted of denaturation at 92ºC for 15 sec, annealing and primer extension at 60ºC for 1 min. The post-read was performed at 60ºC for 1 min. Cases with undetermined genotype even after a second round were excluded. Likewise, in each 96-well plate negative controls were included and ten per cent of genotyped samples were randomly selected for a second analysis.

3.2.5. Statistical analysis

The Hardy–Weinberg equilibrium was tested by a Pearson goodness-of-fit test to compare the observed versus the expected genotype frequencies.

Data analysis was performed using the computer software *Statistical Package for Social Sciences-SPSS* for Windows (version 15.0). Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Statistical differences between mean values were evaluated applying the Mann–Whitney test.

Multivariate logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between variant allele carriers and the risk for the development of colorectal cancer. The potential confounding variables: age, gender, BMI and smoking habits were addressed either by being included as covariates in the multivariate analysis or/and through data stratification.

Homozygotes for the allele with the highest frequency were used as the reference group for each OR estimation. Variant allele carriers were defined as the heterozygous and minor allele homozygous genotype carriers pooled together (dominant model).

Cumulative hazard function plots were estimated by the Kaplan–Meier method with the log-rank test in order to compare groups. We considered the waiting time for the onset of

CRC as the interval between the initial exposure (birth) to the risk factor (*COX-2* genotypes) and the time of onset of disease (age at diagnosis).

3.3. RESULTS

3.3.1 Description of participants

The characteristics of the study population are summarized in Table 2. Cases were significantly older than controls' with a median age of 61 years [50-75] (vs 55 years in controls [50-65], $P<0.001$).

Table 2. Description of participants

	Cases (n=117)	Controls (n=256)	*P*
Demographics			
Age, years			
Mean (SD)	62 (4.1)	56 (6.9)	
Median [min-máx]	61 [50-65]	55 [50-75]	<0.001§
Gender, n (%)			
Male	74 (63.2)	162 (63.3)	0.995
Female	43 (36.8)	94 (36.7)	
Lifestyle behaviors‡			
BMI, Kg/m²			
Mean (SD)	28 (4.0)	28 (3.6)	
Median [min-máx]	28 [20-43]	27 [21-40]	0.643§
BMI category†, n (%)			
<25	21 (18.3)	40 (24.1)	0.243
≥25	94 (81.7)	126 (75.9)	
Smoking status, n (%)			
Never smokers	68 (58.1)	104 (65.4)	0.217
Ever smokers	49 (41.9)	55 (34.6)	
Tumor characteristics¥			
Tumor location, n (%)			
Recto	44 (37.6)	-	
Colon	73 (62.4)	-	
Stage, n (%)			
I-II	57 (48.7)	-	
III-IV	60 (51.3)	-	
Grade, n (%)			
Low/Intermediate	88 (75.2)	-	
High	5 (4.3)	-	
Undefined	24 (20.5)	-	
Synchronous tumors, n (%)			
Yes	7 (6.0)	-	
No	110 (94.0)	-	

BMI, body mass index; †, categorization based on the cut-off defined by WHO for overweight people; ‡, the numbers may not add-up since we were unable to gathered this information for all subjects, namely in controls' group; ¥, for synchronous tumors the most advanced lesion was the one considered in tumors' characterization; §, *P*-value was estimated using the non-parametric Mann-Whitney test.

There were no significant differences in gender, BMI and smoking habits distribution between both groups, although the lifetime exposure to cigarette smoke was higher is cases (37 vs 23 packs-years, *P*=.002) (data not shown) supporting the growing evidences for a role of cigarette smoke in colorectal carcinogenesis.[24-26] Males represented 63% of either populations (*P*=0.995) and 82% of cases were overweight (vs 76% in controls, *P*=0.243).

3.3.2 Genotype frequencies and risk estimates

Genotypes' frequency of each *COX-2* polymorphisms according to disease status is displayed in Table 3.

Table 3. Genotype frequencies among cases and controls and univariate and multivariate *Odds Ratio* (95% CI) estimation on the role of *COX-2* polymorphisms in CRC onset

	Univariated Analysis					Multivariated Analysis			
Polymorphism	Cases	Controls	OR	95% CI	*P*	N†	aOR	95% CI	*P*
-1329A>G									
AA	70 (59.8)	177 (69.1)	1.00	Reference	-		1.00	Reference	-
AG	43 (36.8)	73 (28.5)	1.489	0.933-2.377	0.094	267	1.673	0.964-2.903	0.067
GG	4 (3.4)	6 (2.3)	1.686	0.462-6.155	0.425	187	3.170	0.468-21.48	0.237
G carriers	47 (40.2)	79 (30.9)	1.504	0.955-2.371	0.078	272	**1.735**	**1.011-2.975**	**0.045**
-899G>C									
GG	77 (65.8)	166 (64.8)	1.00	Reference	-		1.00	Reference	-
GC	38 (32.5)	83 (32.4)	0.987	0.617-1.578	0.956	265	0.862	0.500-1.486	0.592
CC	2 (1.7)	7 (2.7)	0.616	0.125-3.034	0.548	179	0.359	0.060-2.161	0.263
C carriers	40 (34.2)	90 (35.2)	0.958	0.605-1.518	0.856	272	0.812	0.477-1.383	0.444
***429T>C**									
TT	54 (47.0)	118 (46.1)	1.00	Reference	-		1.00	Reference	-
TC	51 (44.3)	114 (44.5)	0.978	0.616-1.550	0.923	248	0.869	0.510-1.480	0.604
CC	10 (8.7)	24 (9.4)	0.910	0.407-2.036	0.819	147	0.858	0.314-2.347	0.766
C carriers	61 (53.0)	138 (53.9)	0.966	0.621-1.501	0.878	270	0.874	0.525-1.457	0.606

†total number of participants included in the multivariated analysis; aOR- adjusted *Odds Ratio* (OR) for age, gender, body mass index (BMI) and smoking status; CI- confidence interval.

The genotypic distribution of all three single nucleotide polymorphisms (SNP) in the control group was in agreement with the HWE principles (*P*≥0.05) and the frequencies for the -1329G, -899C and *429C alleles were 17, 19 and 32%, respectively. No significant differences in genotype distribution were noticed for the *COX-2* genetic variations addressed, although, the heterozygous -1329AG genotype was overrepresented in cases' (36.8 vs 28.5%, *P*=0.094) with G allele carriers showing an increased risk trend for CRC onset (OR=1.50; 95%CI:0.955-2.371, *P*=0.078) that reach the significance level in a

multivariate analysis addressing potential confounders for CRC (age, gender, BMI and smoking habits). The susceptibility for CRC development was enhanced by nearly two-folds in individuals carrying the -1329G allele (OR=1.74; 95%CI:1.011-2.975, *P*=0.045).

3.3.3. Gene-environment interaction

Upon a stratified analysis we observed a measurable interaction between the -1329AG genotype and gender (OR=2.49; 95%CI:1.237-5.028, *P*=0.001 in males) or smoking habits even though we were only able to gather information about the smoking status in 62% of controls (OR=8.15; 95%CI:2.658-24.99, *P*<0.001 in ever-smokers) (data not shown). These increased-risk associations were further accentuated in males or ever-smokers -1329G allele carriers (OR=2.58; 95%CI:1.290-5.154, *P*=0.007 and OR=10.27; 95%CI:3.374-31.24, *P*<0.001, respectively), as it can be seen in Table 4.

Table 4. Adjusted *Odds Ratio* (95% CI), under the dominant model, for the influence of *COX-2* polymorphims in CRC onset stratified for age, gender, BMI and smoking status

Stratification		-1329A>G (G carriers vs AA)			-899G>C (C carriers vs GG)			*429T>C (C carriers vs TT)		
	N	aOR	95% CI	*P*	aOR	95% CI	*P*	aOR	95% CI	*P*
Age (years)										
<57	125	1.423	0.604-3.353	0.419	1.577	0.688-3.617	0.282	1.336	0.577-3.093	0.499
≥57	147	1.933	0.946-3.952	0.071	0.552	0.270-1.131	0.104	0.736	0.376-1.438	0.369
Gender										
Female	97	0.632	0.243-1.643	0.346	0.923	0.345-2.467	0.872	0.623	0.252-1.541	0.306
Male	175	**2.579**	**1.290-5.154**	**0.007**	0.854	0.441-1.651	0.638	1.060	0.559-2.011	0.585
BMI (Kg/m²)										
<25	57	2.479	0.686-8.956	0.166	0.366	0.106-1.270	0.113	0.549	0.160-1.884	0.341
≥25	215	1.608	0.881-2.934	0.122	0.959	0.525-1.750	0.892	0.959	0.543-1.695	0.886
Smoking status										
Never smokers	169	0.612	0.296-1.264	0.185	1.003	0.488-2.062	0.994	0.904	0.460-1.775	0.769
Ever smokers	103	**10.27**	**3.374-31.24**	**<0.001**	0.789	0.340-1.829	0.581	0.958	0.417-2.205	0.920

aOR, adjusted *Odds Ratio* (OR) for age, gender, body mass index (BMI) and smoking status; CI, confidence interval

To further explore these associations we analysed the interaction between all three variables (gender, smoking status and genotype), as shown in Table 5. The susceptibility for CRC seemed to be particularly modulated by the presence of the G allele but only in males who ever-smoked (OR=9.01; 95%CI:2.940-27.64, *P*<0.001), although we should be careful

as only 11 out of 104 participants (11%) that were former or current smokers were women. A risk model for CRC was defined considering these individuals and then tested by comparing them to all other participants and as observed in the bottom of Table V a nearly 8-fold higher predisposition for CRC was detected albeit only 31 individuals were included in this model.

Table 5. Multivariate analysis assessing the interaction between gender, smoking status and -1329G>A *COX-2* polymorphism in CRC onset.

	Cases n (%)	Controls n (%)	aOR	95% CI	*P*
Females					
Never-smokers					
AA	27 (65.9)	28 (58.3)	1.00	Reference	-
G carriers	14 (34.1)	20 (41.7)	0.484	0.177-1.325	0.158
Ever-Smokers					
AA	1 (50.0)	9 (100)	1.00	Reference	-
G carriers	1 (50.0)	0 (0)	-	-	-
Males					
Never-smokers					
AA	20 (74.1)	38 (67.9)	1.00	Reference	-
G carriers	7 (25.9)	18 (32.1)	0.71	0.239-2.096	0.533
Ever-smokers					
AA	22 (46.8)	40 (87.0)	1.00	Reference	-
G carriers	25 (53.2)	6 (13.0)	**9.02**	**2.940-27.64**	**<0.001**
Risk Model					
Not†	92 (78.6)	153 (96.2)	1.00	Reference	**-**
Males ever-smokers G allele carriers	25 (21.4)	6 (3.8)	**7.75**	**2.918-20.58**	**<0.001**

aOR, adjusted *Odds Ratio* (OR) for age, gender, body mass index (BMI) and smoking status; CI, confidence interval; †, not "Males ever-smokers G allele carriers"

No association was noticed for the remaining two SNPs in the categorized analysis. In addition, no difference in genotypes' distribution was notice considering the clinicopathological variables (data not shown).

In Figure 1 is shown the cumulative probabilities for CRC occurrence in function of the time to onset of the disease. The waiting time for CRC occurrence was remarkably lower, once again, in men G allele carriers who ever-smoked (62 vs 69 years, Log Rank $P<0.001$).

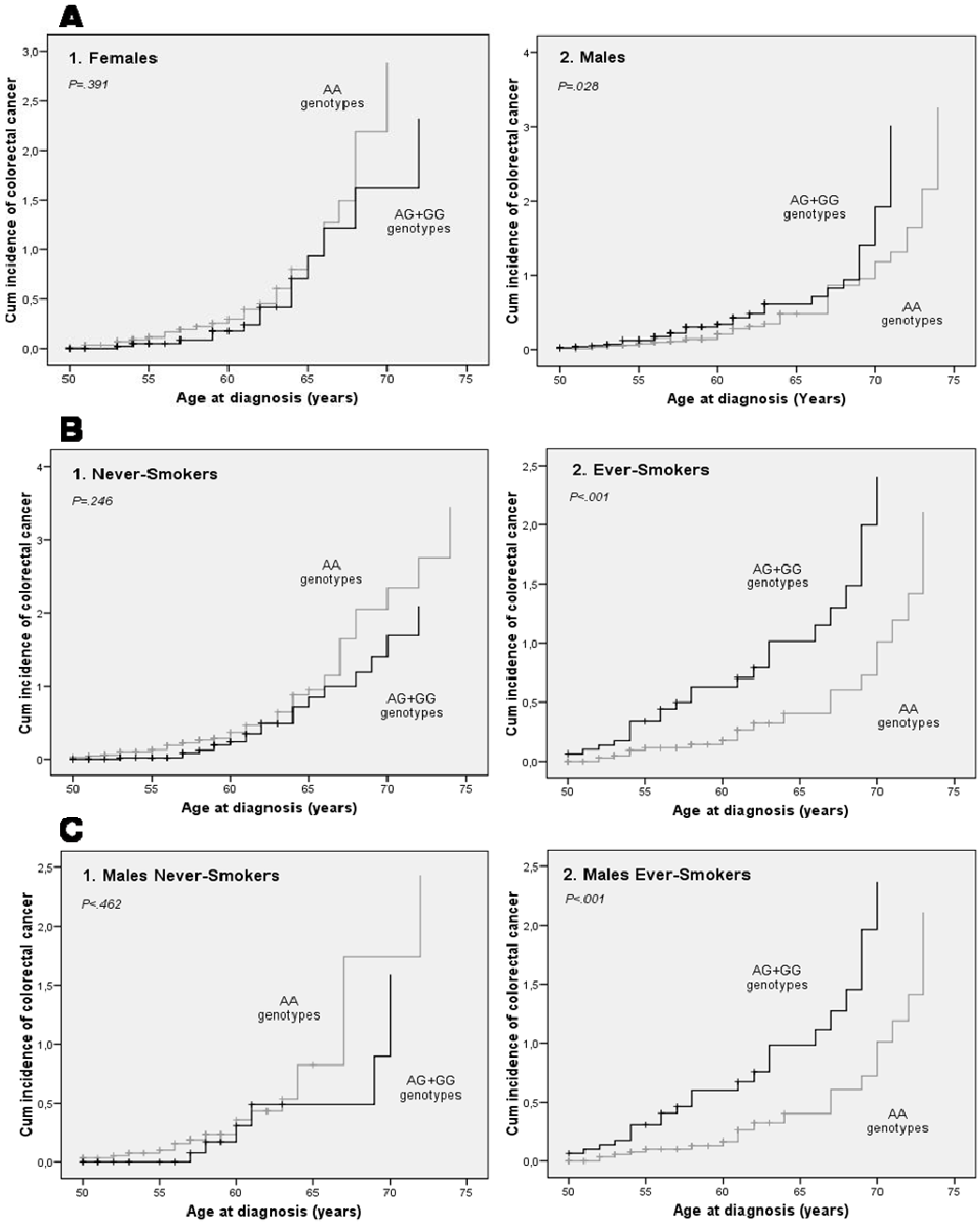

Figure 1. Association of -1329A>G and the waiting time to onset of disease. Cumulative hazard function plots by the Kaplan–Meier methodology and log rank test. Statistical analysis stratified by: (A) gender. In males (A2): 67 vs 66 years (AA and AG+GG genotypes, respectively, P=0.028); (B) smoking status. In ever-smokers (B2): 69 vs 61 years (AA and AG+GG genotypes, respectively, P<0.001) and (C) gender and smoking status. In males who ever-smoked (C2): 69 vs 62 years (AA and AG+GG genotypes, respectively, P<0.001).

3.4. DISCUSSION

CRC, although being the most susceptible GI malignancy to effective prevention is still the second most frequent cancer in developed countries, only behind lung cancer and accounting for over 13% of all incident cases in 2002.[1]

Early screening for colorectal tumors in average-risk individuals is the cornerstone of CRC prevention that is often limited by the low compliance with screening guidelines.[27] COX-2 is considered a biomarker as its behaviour closely resembles colorectal carcinogenesis, being normally undetectable in physiological conditions and increasingly up-regulated with lesions' progression from colorectal adenoma (50%) to cancer (85%).[14] Although the regular use of NSAIDs in cancer prevention is currently jeopardized by the adverse GI complication,[16] the anti-inflammatory and anti-tumor protective effects of their use, through inhibition of COX-2, are undeniable and consistently observed in several epidemiological and clinical trials.[8-10]

In this case-control study we assessed the role of -1329A>G, -899G>C and *429T>C polymorphisms, expected to modulate COX-2 expression profile, in CRC development not disregarding a possible gene-environment interaction.

All three functional expected polymorphisms were previously addressed for their involvement in GI tumors susceptibility although only in a restricted number of studies as reviewed in a recent study.[18]

The -899G>C promoter region polymorphism was associated with a higher CRC predisposition in Asiatic populations[18], although the positive association was only detected in the study by Tan *et al.*[28] that gathered 2300 participants and not reproducible in the remaining three studies with smallest populations,[29-31] suggesting not a lack of influence but most certainly a low statistical power to detected a small difference in genotypes' frequency between groups. We failed to show an association between this polymorphism and CRC risk in our population that overlaps with the only study previously assessed for CRC risk in Caucasians.[32] In contrast with the increased risk observed for CRC, Ueda *et al.*,[33] in a males only study, reported a nearly 50% protection for colorectal adenomas onset that was highlighted among -899CC genotype carriers non-users of aspirin and other NSAIDs in the study developed by Ulrich *et al.*,[34] in which the protection was enhanced to 70%. Furthermore, in the study reported by Xing *et al.,*[29] and not disregarding the general non-

association observed between -899G>C genotypes and CRC risk, an interaction with smoke consumption and alcohol drinking was observed in -899GG genotype carriers. In our study and although we had enough statistical power to detect the same two-fold increased risk we did not observe any association upon a smoking habits stratified analysis. The dual and antagonic influence that this polymorphism seems to play in tumor development could have a biological reasoning as the presence of allele C in *COX-2* promoter region, in one instance eliminates the recognition binding site for the Sp1 positive transcription factor leading to a 30% reduction in promoter's activity *in vitro*[19] on the other hand it also creates an E2F homology binding region that could lead to a higher transcription activity.[20] In fact, the -899CC genotype was associated with a 10-fold enhanced production of PG, compared to the homozygous -899GG,[35] thus supporting the observed increased susceptibility to several GI malignancies.

Given its location in the 3'UTR of *COX-2* gene the *429T>C polymorphism is a potential candidate to modulate the genetic predisposition to CRC. The thymine (T) to cytosine (C) exchange in an AU-rich elements region, known to control mRNA stability and degradation of several other early-immediate genes encoding inflammatory mediators whose mRNA is very unstable,[36] could enhance the mRNA transcripts stability and ultimately lead to an increased prostaglandins production. This genetic variation has been previously shown to influence the development of several malignancies, like breast and lung carcinomas.[37, 38] In the GI tract the *429C allele as been associated with a 1.25-fold increased susceptibility for colorectal adenoma and paradoxically with a protective effect for gastric cancer, compared with *429GG genotype.[18] Overall, the involvement of this SNP in colorectal cancer, however, could not be demonstrated in this study, which is in agreement with the already published studies[32, 39] with the more recent gathering over 2000 Caucasian participants from France[40]. These findings appear to suggest that the *429T>C polymorphism is more important in early stages of colorectal tumor formation but not so relevant in the progression from adenomatous polyps to malignant tumors.

When focusing on -1329A>G *COX-2* promoter region polymorphism we observed an overrepresentation of AG and GG genotypes in the group of cases that translated in a 1.7-fold increased predisposition to CRC onset in G allele carriers upon a multivariate analysis. Our results were rather unexpected, although two previous studies had shown some tendency towards our findings. Ueda *et al.*[33] in a Japanese study (Self Defense Forces Health Study) involving male officers reported an inverse association between the AA genotype and the genetic susceptibility for colorectal adenoma, although the 95%CI intercept the unit. Likewise, in a case-control study conducted in Caucasians, Siezen *et al.*[41] reported an increased risk trend when comparing the homozygous GG vs AA genotypes for their role in adenoma onset. These associations have raised some controversy as they cannot be biologically interpreted considering the previous functional study developed by Zhang *et al.*[20] In this study the G to A substitution in *COX-2* promoter region created a recognition binding site for the c-MYB nuclear protein resulting in a higher transcriptional activity and in an increased *COX-2* mRNA expression that could be translated in a significantly higher risk for esophageal cancer in -1329AA genotype carriers.[20] This biological plausibility had further repercussions in several epidemiological studies, being the AA genotype associated with a higher genetic predisposition for the development of esophageal, gastric and colorectal adenocarcinomas in Asiatic populations.[20, 28, 42, 43] Overall, these contradictory associations appear to suggest the involvement of other factors that could influence this polymorphism behaviour possibly through gene-environment interactions that can modulate cellular microenvironment and thus the binding of different nuclear proteins to *COX-2* promoter region.

To explore this hypothesis we carried-out a stratified analysis considering several risk factors known to contribute to CRC development and observed a positive association between -1329AG/GG genotypes and CRC in males and in ever active smokers and more interestingly a 9-fold increased predisposition in males who ever-smoked. The lack of association in females might be attributable to the small sample size of this group and to

the fact that only 11% (n=11) of women were current or former-smokers in the three variable interaction analysis. If, otherwise confirmed by larger studies, this could possibly be explained since both genders might have different lifestyle habits or be exposed to different environmental factors not considered in this multivariate analysis. Tobacco smoke, although not always consistently, as been implicated in the colorectal carcinogenesis[25, 26] and COX-2 overexpression is suggested as one of the smoke-induced pathways involved in tumor development.[44, 45] Tobacco contains over 60 identified carcinogens and even though some, such as, nicotine and benzo[a]pyrene, where shown to trigger COX-2 expression via β-adrenoceptors and ERK1/2 pathways, respectively, the pathogenesis of smoking-related colorectal cancer is still understudied.[45, 46] Hence, it is possible that other smoke-induced pathways may interact with -1329G allele containing promoter region leading to a differential COX-2 expression that only future *in vitro* studies will elucidate. In addition, COX-2 besides being triggered by benzo[a]pyrene metabolizes it to benzo[a]pyrene diolepoxide, a powerful carcinogen when carrying out its peroxidase activity[47] thus suggesting a feedback mechanism capable of magnifying the smoke-induced carcinogenic pathway promoted by this xenobiotic, more noticeably in individuals expected to express higher levels of COX-2 protein.

Considering that COX-2-derived PGE-2, the most abundant prostaglandin in colorectal tumors, is implicated in key steps of tumor development including inhibition of apoptosis, stimulation of angiogenesis, tumor proliferation and immunosupression,[13] it is plausible that functional polymorphisms in *COX-2* gene might influence the time-to-onset for CRC by modulating COX-2 expression and thus the exposure to PGE-2-induced proneoplastic effects. In fact, we observed that the diagnosis of CRC was anticipated by seven years in male cases carriers of -1329G allele who ever consumed tobacco once again reinforcing the role of this *COX-2* SNP in colorectal neoplasia.

These findings represent a preliminary study and as so there are some drawbacks that need to be considered in their interpretation. A major limitation is the missing information on

BMI and smoking status in control's group. We only had data in approximately 62% of population, thus explaining the wider 95%CI observed in the gene-environment interaction analysis. We also cannot exclude selection bias as only 43% of eligible cases were interviewed and informed of this study, mostly explained by the work overload of the clinicians. The sample size of our population, especially the cases' group, is insufficient to detect low magnitude associations and to allow conclusive findings in the stratified analysis. Although we imposed the same age-restriction criteria we notice that CRC patients were significantly older than controls'. We included age as a covariate in the multivariate analysis. This statistical adjustment strengthened rather than weakened our findings.

A clearer understanding on CRC etiology through the identification of risk factors might allow a better definition of risk models that are more likely to benefit from preventive strategies. Chan *et al.*[15] reported that the chemopreventive effects of regular use of aspirin on CRC development were exclusively effective in COX-2 overexpressing tumors. In this study we observed that males who ever-smoked carrying at least one -1329G allele had not only a higher genetic predisposition but also were expected to develop CRC at an earlier age, suggesting that this SNP may help predict individuals expected to be exposed to higher levels of COX-2 and thus susceptible to be defined as a risk model for CRC development as observed in this study.

Further research is needed to corroborate our results, namely larger observational studies in association with COX-2 expression reports in CRC tumors, to subsequently determine whether this specific subgroup of "higher-risk" individuals would in reality benefit from the use of COX-2 inhibitors by shifting the balance between cost and benefits that is currently overshadowed by the adverse GI and cardiovascular side-effects.

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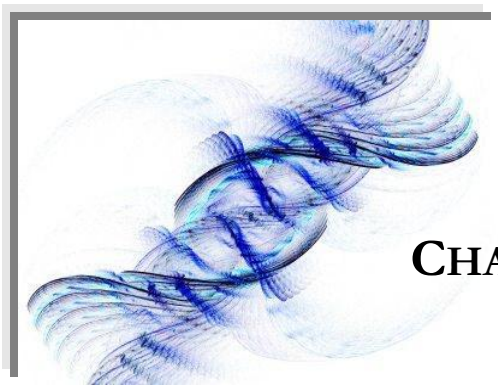


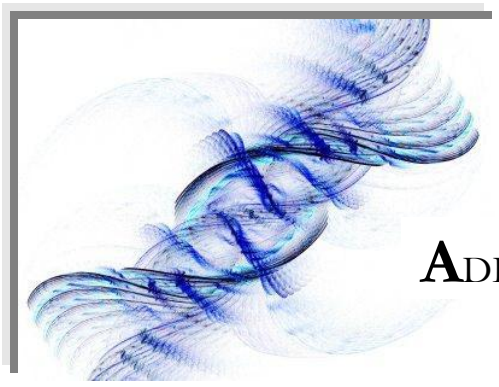
CHAPTER IV

FUTURE STUDIES

In this study we noticed that men who ever-smoked, simultaneously -1195G allele carriers, expected to be exposed to higher COX-2-derived PGE_2 levels, might be at increased risk and represent a risk model for CRC development. Before this finding could have clinical translation further research should be warranted in this field, namely:

- Its reproduction in larger epidemiological studies incorporating, also, other polymorphisms in genes that may influence PGE_2 levels and signaling pathways in a gene-gene interaction and haplotypic analysis. Although, the steady-state level of PGE_2 is maintained in the tumor microenvironment by a balance between biosynthesis and degradation, virtually, all reports assessing its role in cancer have focused exclusively on the COX-dependent biosynthesis. By example, 15-hydroxyprostaglandind dehydrogenase (15-PGDH) catalyzes the rate limiting step of PGE_2 catabolism and has been shown to be downregulated in CRC, so it provides a potential mechanism for local accumulation of PGE_2;
- *In vitro* studies to characterize functionally the interaction between the -1329A>G polymorphism in the promoter region of *COX-2* and smoke-induced colorectal carcinogenesis in association with COX-2 protein expression studies in CRC tumors to corroborate the observed increased susceptibility.

Furthermore, COX-2 overexpression is already detected in 40-50% of colorectal adenomas and its inhibition by regular use of COX-2 inhibitors has been associated with protection for both polyp incidence and recurrence. In agreement, this study should be extended to patients with previous adenomas, not overlooking the occurrence of metachronous lesions. Ultimately, this could allow a more comprehensive understanding of the contribution of both genetic and environmental factors in colorectal carcinogenesis.



ADDENDA

Scale for Quality Assessment	
Criteria	Score 0 to 50
Title and Abstract: Is the article identified as an observational study (case-control or cohort study) in the title or abstract? (1 - yes; 0 - no)	□ 0 □ 1
Title and Abstract: Is the abstract informative and structured? (1 - yes; 0 - no)	□ 0 □ 1
Introduction: Does the introduction explain scientific background and the rationale for the research being reported? (1 - yes; 0 - no)	□ 0 □ 1
Introduction: Does the introduction include a clear statement of objectives? (1 - yes; 0 - no)	□ 0 □ 1
Methods / Study design: Does the article present all key elements of study design (including designation and correctness of study designation)? (2 - yes; 0 - no)	□ 0 □ 2
Methods / Setting: Does the article describe setting (includes locations and dates for data collection)? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are inclusion and exclusion for cases clear stated? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are methods of selection described? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are clear diagnostic criteria stated for cases (in case-controls)? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are cases based on histological confirmation (ascertainment of cases)? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Are cases adequately representative (selection bias)? (2 – From cancer registries; 1 – From clinical databases; 0 – w/o clearly defined sampling frame)	□ 0 □ 1 □ 2
Participants: Is the rationale for controls stated? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Are sources of controls adequate? (3- population or neighbour-based; 2- blood donors; 1- hospital based or healthy not-stated; 0- not described)	□ 0 □ 1 □ 2 □ 3
Participants: Are matching criteria for controls stated? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Are controls histological based (ascertainment of cases)? (2 - yes; 0 - no)	□ 0 □ 2
Variables of interest: Are all outcomes variables adequately described? (1 - yes; 0 - no)	□ 0 □ 1
Bias: Were potential confounders or effect modifiers adequately taken care? (2 - yes; 0 - no)	□ 0 □ 2
Measurements: Are details of methods of measurements given? (1 - yes; 0 - no)	□ 0 □ 1
Measurements: Are measurements performed equally between cases and controls? (2- yes; 0 - no)	□ 0 □ 2
Bias: Are measurements of exposure made blinded/independently measured to group (in case-controls) or definition of outcome blinded to exposure (in cohorts)? (2 - yes; 0 - no)	□ 0 □ 2
Hardy-Weinberg equilibrium: Was HWE assessed? (1 - yes; 0 - no)	□ 0 □ 1
Hardy-Weinberg equilibrium: Were control populations in HWE? (1 - yes; 0 - no)	□ 0 □ 1
Sample size: Are sample size estimates described? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: Were methods used to assess association between exposure (polymorphisms) and outcome (cancer) adequate? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: was management of confounders taken care (subgroups analysis)? (1 - yes; 0 - no)	□ 0 □ 1
Statistical methods: Was missing data managed in case-controls? (1 - yes; 0 - no)	□ 0 □ 1
Or	
Bias: In cohort studies, was the follow-up more than 80%? (1 - yes; 0 - no)	□ 0 □ 1
Funding and disclosure statement: Are funding and/or disclosure clearly stated?	□ 0 □ 1
Participants: Does the manuscript reports adequately the numbers of individuals at each stage of study (eligible, participating and analysed)? (3 - yes; 0 - no)	□ 0 □ 3
Participants: Are reasons of non-participation presented? (1 - yes; 0 - no)	□ 0 □ 1
Participants: Does a flow diagram presented? (1 - yes; 0 - no)	□ 0 □ 1

Scale for Quality Assessment (continued)	
Criteria	Score 0 to 50
Internal validity: Are selected and participants similar in important variables? (2 - yes; 0 - no)	□ 0 □ 2
Descriptive data: Are characteristics of participants described (eg, demographic, clinical, social)? (1 - yes; 0 - no)	□ 0 □ 1
Outcome data: Does the manuscript describe adequately the numbers of outcomes events in exposed, or, of exposition in cases and controls? (1 - yes; 0 - no)	□ 0 □ 1
Main results: Are measures of association presented with precision estimates (95% CI)? (1 - yes; 0 - no)	□ 0 □ 1
Main results: Are measures of association adjusted to confounders? (2 - yes; 0 - no)	□ 0 □ 2
Discussion: Does the discussion include a summary of main results? (1 - yes; 0 - no)	□ 0 □ 1
Limitations: Are limitations discussed? (1 - yes; 0 - no)	□ 0 □ 1
Generability and interpretation: Does the manuscript discuss generability? (1 - yes; 0 - no)	□ 0 □ 1

Polymorphisms characterization						
Polymorphism § (other names Ω)	dbSNP # (rs number)	Nucleotide position Φ	Nucleotide alteration ¥	amino acid alteration	Biological plausibility	Function
COX-1						
Intronic region						
IVS7+14delA	rs3215925	11707	Deletion of A (intron 7)	-	Unknown	Unknown
IVS7-45T>C	rs3842798	13410	g.12380 (intron 7)	-	Unknown	Unknown
Coding region						
L15_L16del	(1)	-	-	Deletion of two Leucines (L) in the COX1 signal peptide region	Probably has little impact in protein location[34]†	Unknown
p.R8W	rs1236913	1149	g.116C>T (exon 2)	Arginine (R)-to-Tryptophan (W) at a.a.8	Probably affect protein function[76]†; may alter the binding of putative splicing factors (SR proteins)[77]†	Normal expression[83]‡
p.P17L	rs3842787	1176	g.144C>T (exon 2)	Proline (P)-to-Leucine (L) at a.a.17	Probably affect protein function[76]†; may alter the binding of putative splicing factors (SR proteins)[77]†	Normal expression[83]‡
p.Q41Q	rs3842788	7873	g.6843A>G (exon 3)	Glutamine (Q)-to-Glutamine at a.a. 41	Silent polymorphism	Unknown
p.G213G	rs5788	11459	g.10429C>A (exon 6)	Glycine (G)-to-Glycine at a.a.213	Silent polymorphism	Unknown
p.L237M	rs5789	11640	g.10608C>A (exon 7)	Leucine (L)-to-Methionine (M) at a.a.237	Located near the COX-1 dimer interface connecting two identical monomeric subunits (new hydrogen bond);[76,82] Probably affect protein function[76]†	Decreased activity[83]‡
p.V481I (V444I)	rs5794	20287	g.19255G>A (exon 10)	Valine (V)-to-Isoleucine (I) at a.a.481	Unknown	Unknown
COX-2						
Promoter region						
-1462_-1461delTG (-663GT/(GT))	rs689464	362	Deletion of TG	-	Not in transcription factors binding sites[36]†	Unknown
-1423A>G (-1290A>G, 401)	rs689465	401	g.-1424A>G	-	Unknown	Unknown
-1329G>A (-1195G>A)	rs689466	496	g. -1329G>A	-	Creation of a C-MYB binding site[28]†	Higher transcription activity of the *COX-2* gene[28]‡
-899G>C (-765G>C, 926)	rs20417	926	g.-899G>C	-	Disruption of Sp1 binding site[69] OR possible creation of E2F binding site that may enhance COX-2 expression[70]†	30% reduction in the promoter activity[69]‡; 10-fold increased production of PGE_2 [70]
-798A>G	(2)	-	A>G	-	Not in transcription factors binding sites[36]†	Unknown
-646C>T	rs20420	1179	g-646C>G	-	Unknown	Unknown

Polymorphisms characterization (continued)						
5' UTR (exon 1)						
-196C>G (1629)	rs20424	1629	g.-196C>G	-	Unknown	Unknown
-125T>G (10T>C)	rs5271	1700	g.-125T>G	-	Unknown	Unknown
Intronic region						
IVS5-275T>G (5229, 5209)	rs20432	5209	g.3385T>G (intron 5)	-	Unknown	Unknown
IVS7+111T>C	rs4648276	6043	g.4219T>C (intron 7)	-	Unknown	Unknown
Coding region						
p.V102V (Ex3-8G>C, 3050)	rs5277	3050	g.1226G>C (exon 3)	Valine (V)-to-Valine (V) at a.a.102	Silent polymorphism	Unknown
p.V511A	rs5273	7763	g.5939T>C (exon 10)	Valine (V)-to-Alanine (A) change at a.a.511	Lies inside a tighly packed hydrophobic pocket adjacent to the cyclooxygenase active site[39]	No alteration in enzyme kinetic parameters (Vmáx and Km) or the stability for the utilization of arachidonic acid[39]‡
p.G587R (Gly587Arg)	rs3218625	7990	g.6166G>A (exon 10)	Glycine (G)-to-Arginine (R) change at a.a.587	Unknown	Unknown
3'UTR (exon 10)						
*429T>C (2242T>C; 8494T>C; 8473 Ex10+837C>T)	rs5275	8473	g.6649T>C	-	SNP located in the AU-rich region that mediates transcript degradation that might have a transcript-stabilising function[36,80]	Unknown
***1806A>G** (3618A>G; 9850)	rs4648298	9850	g.8026A>G	-	May have some effect on the addition of some poly-A tail to the mRNA, or can cause a later poly-A site to be used, creating a longer, and possibly more stable species of mRNA[42]	Unknown
***2291G>A** (10335)	rs689469	10335	g.8511G>A	-	Downstream of the last polyadenylation site[42]	Unknown
***2430C>T**	rs689470	10474	g.8650C>T	-	Unknown	Unknown

§- polymorphism designation adopted in our study following the nomenclature system proposed by den Dunnen *et al.*[62]; Ω- other polymorphisms designations found across the selected studies; #- dbSNP (Single Nucleotide Polymorphism database) reference number; Φ nucleotide position on GenBank accession number: AF440204 (*COX-1*) and D28235 (*COX-2*); ¥- nucleotide position relative to gene transcriptional start site †- based on Bioinformatics programs; ‡- observed in *in vitro* studies; a.a.- amino acid; UTR- Untranslated region; (1)- rs number not available; (2)- the rs number presented by the authors wasn't in the dbSNP. An email was sent to the authors[36] to confirm this rs number but no reply was obtained.

Estudo Científico:

"CARACTERIZAÇÃO FARMACOGENÓMICA DA COX NA CARCINOGÉNESE DO CÓLON"

Consentimento informado

Eu, _____, portador do processo clínico do Instituto Português de Oncologia – Núcleo Regional do Porto, nº _____ :

• Fui informado das razões do estudo e das dúvidas que este projecto pretende esclarecer.

• Fui informado dos procedimentos clínicos, endoscópicos e laboratoriais necessários

• Se aceitar participar neste estudo:

- colher-me-ão sangue para análises para determinação no sangue dos polimorfismos genéticos dos genes *COX1* e *COX2*;
- ser-me-ão efectuadas colonoscopias de acordo com o programa de vigilância pré-determinado pelo meu médico assistente;
- durante a realização da endoscopia poderão ter que ser efectuadas biopsias ou polipectomia para caracterização histológica e molecular.

• Fui informado dos riscos desses procedimentos.

• Fui informado que os dados deste estudo pretendem contribuir para a:

- optimização do rastreio de CCR em indivíduos assintomáticos;
- optimização do seguimento de doentes que realizaram colonoscopia que revelou a presença de lesões definidas como pré-neoplásicas (pólipos) ou neoplásicas (CCR).

• Fui informado que os dados deste estudo são confidenciais e podem ser retirados da base de dados, a qualquer altura, caso seja essa a minha decisão.

• Aceito que me colham sangue e que realizem colonoscopia com eventuais biopsias ou polipectomia, participando dessa forma no estudo.

• Só autorizo que os dados decorrentes do estudo sejam os descriminados; outros estudos apenas serão realizados sob minha expressa autorização.

Doente ou representante legal:

Assinatura: _____

Nome: _____

Porto,_____de_____de_____

Responsável pela recolha deste documento:

Assinatura: _____

Nome: _____

Porto,_____de_____de_____

Investigador responsável:

Assinatura: _____

Nome: _____

Porto,_____de_____de_____

Estudo Científico:

"CARACTERIZAÇÃO FARMACOGENÓMICA DA COX NA CARCINOGÉNESE DO CÓLON"

Documento informativo

A. O que é este documento?

Este documento consiste num convite para participar no estudo "Caracterização farmacogenómica da COX na carcinogénese do cólon", e que irá fornecer-lhe informação que o ajudará a decidir se gostaria ou não de participar. Caso decida participar ser-lhe-á entregue uma cópia deste documento.

B. Qual o objectivo deste estudo?

As neoplasias do tubo digestivo são a principal causa de morte por cancro em Portugal. O prognóstico dos doentes está claramente relacionado com o estádio à data do diagnóstico. Estratégias de prevenção poderão representar os meios mais eficazes reduzindo a incidência e mortalidade. Através do rastreio em indivíduos assintomáticos ou pelo seguimento de indivíduos de alto risco poderá ser possível o diagnóstico precoce destas situações.

O objectivo deste estudo é determinar se existe um perfil genotípico que permita identificar pacientes com maior susceptibilidade para o desenvolvimento de lesões colo-rectais e optimizar o seguimento de pacientes com lesões pré-neoplásicas colo-rectais.

C. Quem está a promover este estudo?

O Serviço de Gastrenterologia e o Grupo de Oncologia Molecular do IPO do Porto são os grupos responsáveis por este estudo. Este estudo tem o apoio da Sociedade Portuguesa de Gastrenterologia e foi aprovado pela Comissão de Ética do IPO Porto.

D. Porque fui convidado para este estudo?

Vimos desta forma convidá-lo a participar neste estudo, dado ter efectuado pelo menos uma colonoscopia, no Serviço de Gastrenterologia, entre 2002 e 2007 e pertencer a um dos seguintes grupos: indivíduos sem lesões colo-rectais (grupo controlo), indivíduos com pólipos adenomatosos (grupo com adenomas) e indivíduos com cancro colo-rectal (grupo com cancro). A sua participação neste estudo é voluntária. Se decidir não participar ou desistir deste estudo, não perderá quaisquer cuidados médicos.

E. Que procedimentos serão efectuados neste estudo?

Solicita-se a sua autorização para a recolha de uma amostra de sangue para o estudo referido. Da amostra de sangue será extraído e analisado o DNA para a pesquisa de polimorfismos no genes *COXs* que possam alterar a susceptibilidade para o desenvolvimento de lesões colo-rectais.

F. Quantas pessoas como eu irão participar neste estudo?

O número exacto de indivíduos a participar neste estudo é desconhecido, mas estima-se que sejam necessários entre 100 a 200 indivíduos em cada grupo. No entanto quanto maior for o número de participantes neste estudo maior é a validade do mesmo.

G. Quanto tempo dura o estudo?

O estudo decorrerá até se ter um número suficiente de amostras de sangue capazes de permitir chegar a uma conclusão viável, estando dependentes do número de doentes que aceitem participar neste estudo.

H. Irei eu, ou terceiros, beneficiar com a minha participação neste estudo?

Poderá não receber benefícios directos pela sua participação neste estudo. Contudo, espera-se que este estudo origine informação que possa conduzir à identificação de indivíduos com maior risco para desenvolverem lesões colo-rectais e consequentemente, a um diagnóstico precoce das mesmas e a uma optimização do seguimento de pacientes com lesões pré-neoplásicas (pólipos adenomatosos).

I. O que irá custar este estudo?

Da sua participação neste estudo não resultará qualquer custo adicional.

J. Como será mantida a confidencialidade dos meus registos?

É garantida pela instituição e responsáveis do projecto a rigorosa confidencialidade de todos os dados registados, reservando-lhe o direito, caso assim o entenda, de pedir, em qualquer momento, a sua remoção, assim como, de todos os dados recolhidos, deste estudo.

K. Quem terá acesso à minha informação médica, caso eu assine este consentimento informado?

O acesso directo aos seus registos será requisitado por representantes autorizados pelo promotor do estudo para verificar a informação obtida no decorrer do estudo.
Ao assinar este Consentimento Informado, autoriza (ou o seu representante legal) o acesso a esta informação confidencial.
A confidencialidade dos seus registos médicos será mantida dentro dos limites permitidos pela legislação aplicável. Se os resultados deste estudo forem publicados, a sua identidade permanecerá confidencial.

L. Se eu concordar em participar neste estudo, que certezas posso ter?

- ✓ Que a sua participação é voluntária e que é inteiramente livre de desistir em qualquer altura, sem que isso ponha em risco a sua assistência médica futura.
- ✓ Que o promotor do estudo pode escolher retirá-lo deste estudo de investigação em qualquer altura.
- ✓ Que pode colocar questões acerca deste estudo em qualquer altura.

Se tiver questões acerca do consentimento informado ou dos seus direitos como doente a participar num estudo de investigação, pode contactar o Instituto Português de Oncologia Francisco Gentil do Porto:

Grupo de Oncologia Molecular
Dra. Carina Pereira
Tlf. 225084000 (ext.5413)

QUESTIONÁRIO CANCRO COLO-RECTAL

1. INTRODUÇÃO:

1.1. Nome: **1.2. Número IPO:**

1.3. Telefone: **1.4. Distrito:**

1.5. Data de nascimento: **1.6. Género:** **1.7. Etnia:**

M ☐ F ☐ Caucasiana ☐ Africana ☐ Asiática ☐

1.8. Profissão:

2. CARACTERIZAÇÃO DE RISCO:

2.1. Peso: **2.2. Altura:** **IMC:**

2.3. História familiar de CCR: **Sim** ☐ **Não** ☐

Se sim, qual o grau de parentesco idade de diagnóstico

Se sim, qual o grau de parentesco idade de diagnóstico

2.4. História familiar de pólipos: **Sim** ☐ **Não** ☐

Se sim, qual o grau de parentesco idade de diagnóstico

Se sim, qual o grau de parentesco idade de diagnóstico

2.5. Antecedentes pessoais de outros cancros: **Sim** ☐ **Não** ☐

Se sim, qual ; ;

2.6. Hábitos tabágicos:

Não fumador ☐

Fumador ☐ idade início cigarros/dia

Ex-fumador ☐ idade início idade fim cigarros/dia

2.7. Actividade física: **Sim** ☐ , quantas horas/semana

Não ☐

No ano anterior ao diagnóstico de pólipos/CCR

3. HÁBITOS DIETÉTICOS:

3.1. Consumo de álcool: **Sim** ☐ **Não** ☐

Vinho ☐ , ____ copos/dia; Cerveja ☐ , ____ copos/dia
Bebidas brancas ☐ , ____ copos/dia; Outra ☐ , ____ ____ copos/dia

3.2. Consumo de fibras:

Diariamente ☐ 2-3x/semana ☐ <2x/semana ☐ ____

3.3. Consumo de carnes vermelhas:

Diariamente ☐ 2-3x/semana ☐ <2x/semana ☐ ____

3.4. Consumo de gorduras:

Diariamente ☐ 2-3x/semana ☐ <2x/semana ☐ ____

3.5. Consumo de suplementos de cálcio, leite, lacticínios:

Diariamente ☐ 2-3x/semana ☐ <2x/semana ☐ ____

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4. USO DE ASPIRINA OU OUTROS AINEs: **Sim** ☐ **Não** ☐

Se sim, qual?

Aspirina ☐	**Ibuprofeno** ☐	**Diclofenac** ☐	**Naproxeno** ☐	**Nimesulida** ☐
☐ Cartia	☐ Brufen	☐ Voltaren	☐ Naprosyn	☐ Aulin
☐ AAS	☐ Trifene	☐ Flameril	☐ Reuxen	☐ Nimed
☐ Migraspirina	☐	☐ Olfen	☐	☐ Sulimed
☐	☐	☐ Fenil-V	☐	☐ Donulide
☐	☐	☐ Cataflam	☐	☐ Vitolide

Outras observações:

DNA PURIFICATION FROM BLOOD PROTOCOL (SPIN PROTOCOL)

- 20 µL of Proteinase K was pipeted into the bottom of a 1.5 mL microcentrifuge tube;
- Next, 200 µL of blood sample was added to the microcentrifuge tube followed by 200 µL of Buffer AL (lysis) and pulse-vortexing for 15s;
- The sample was incubated at 56ºC for 10 min;
- Afterward, a brief centrifugation was conducted to remove drops from the inside of the lid;
- Then, 200 µL of ethanol (96-100%) was added to the sample and another mixture by pulse-vortex was applied for 15s. In order to remove drops from the inside of the lid, centrifugation was carried out once again.

The next steps were performed with extremely care

- The mixture was applied to the QIAamp Spin Column (in a 2 mL collection tube) without wetting the rim and centrifuged at 8000 rpm for 1 min. The QIAamp Spin Column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded;
- 500 µL of Buffer AW1 (wash) was added to the QIAamp Spin Column without wetting the rim and centrifuged at 8000 rpm for 1 min. The QIAamp Spin Column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded;
- Next, 500 µL of Buffer AW2 (wash) was added to the QIAamp Spin Column that was then submitted to a full speed centrifugation (13.2 rpm) for 3 min;
- The final step consisted in adding 200 µL of Buffer AE (elution) to the QIAamp Spin Column placed in a clean 1.5mL microcentrifuge tube. The solution was

incubated at room temperature for 1 min, previously to being centrifuged at 8000 rpm for 1 min.

- The extracted DNA was then conserved at -20ºC until the genotype analysis.