Role of genetic polymorphisms in PTGS2 and HPGD in colorectal cancer development

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pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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RESUMO

O cancro colo-rectal (CCR) é um sério problema de saúde a nível mundial. Em Portugal, o CCR é a segunda causa de morte por cancro em ambos os sexos. A prostaglandina E2 (PGE2) é mediadora de uma grande variedade de efeitos fisiológicos e tem um papel chave na inflamação e no cancro. A concentração de PGE2 no microambiente do tumor depende do balanço entre a sua síntese, protagonizada pela ciclooxigenase-2 (COX-2), e a sua inativação catabólica pela 15-prostaglandina desidrogenase (15-HPGD). A expressão aumentada de COX-2 e a sub-regulação de 15-PGDH têm sido observadas em CCR. O objetivo deste estudo foi avaliar a influência de polimorfismos dos genes *PTGS2* e *HPGD*, que codificam a COX-2 e a 15-PGDH, respetivamente, numa população da região Norte de Portugal. Foi desenvolvido um estudo caso-controlo de base hospitalar envolvendo 798 participantes: 254 pacientes com diagnóstico de CRC e 544 controlos sem evidência de cancro, recrutados no Instituto Português de Oncologia do Porto (IPO Porto). Os polimorfismos do gene *PTGS2* (-1195A>G, -765G>C e 8473T>C) e os polimorfismos do gene *HPGD* (rs8752, rs2612656 e rs2555639) foram caracterizados recorrendo à técnica de discriminação alélica, por PCR em Tempo Real. O polimorfismo -1195A>G apresentou uma predisposição 2.04 vezes superior para o desenvolvimento de CCR. Numa análise estratificada, os homens e indivíduos ex-/fumadores, portadores do genótipo GG, apresentaram um risco aumentado para o desenvolvimento de CCR (OR 3.10; 95% CI: 1.22-7.88; *P*=0,013 e OR 3.90; 95% CI: 1.17-12.96; *P*=0.019, respetivamente). Os portadores do genótipo -765CC apresentaram uma tendência protetora para o desenvolvimento de CCR (OR 0.62; 95% CI: 0.22-1.71; *P*=0.345). Similarmente, o polimorfismo rs2612656A>G associou-se a uma redução no risco para CCR (OR 0.62; 95% CI: 0.43-0.90; *P*=0.011), que foi mais evidente em mulheres, indivíduos não fumadores e obesos portadores do genótipo AG (OR 0.38; 95% CI: 0.19-0.76; *P*=0.005, OR 0.53; 95% CI: 0.28-0.97; *P*=0.039 e OR 0.39; 95% CI: 0.15-0.97; *P*=0.039, respetivamente). Relativamente ao polimorfismo rs8752C>T, portadores do genótipo CC, apresentaram um aumento de 40% de risco para o desenvolvimento de CCR (OR 1.41; 95% CI: 0.90-2.22; *P*=0.133), o qual atinge valores significativos em indivíduos com idade superior a 59 anos (OR 1.94; 95% CI: 1.04-3.61; *P*=0.035). Podemos concluir que os polimorfismos -1195A>G e -765G>C do gene *PTGS2* e rs2612656 e rs8752 do gene *HPGH* parecem influenciar a suscetibilidade genética para CRC. Este é o primeiro estudo a avaliar a associação entre polimorfismos no gene *HPGD* e a suscetibilidade para CCR numa população portuguesa. Os nossos resultados reforçam a importância da via da PGE2 na

carcinogénese colo-rectal mostrando que uma melhor compreensão da etiologia do CCR pode permitir a caracterização de sub-grupos de indivíduos com maior risco, os quais poderão beneficiar de estratégias quimiopreventivas.

Palavras-chave: Cancro colo-rectal; *PTGS2*; *HPGD*; Polimorfismo

ABSTRACT

Colorectal cancer (CRC) is a major health problem worldwide. In Portugal, CRC is the second most common cause of cancer-related death in both genders. Prostaglandin E2 (PGE2) mediates an extensive range of physiological effects and has a key role in inflammation and cancer. The concentration of PGE2 in the tumor microenvironment depends on the balance between its synthesis by cyclooxygenase-2 (COX-2) and its catabolic inactivation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Enhanced expression of COX-2 and down-regulation of 15-PGDH has been observed in CRC. The aim of this study was to evaluate the influence of *PTGS2* and *HPGD* polymorphisms in a population from the Northern region of Portugal. We conducted a hospital-based case-control study with 798 participants: 254 histologically confirmed CRC patients and 544 cancer-free controls recruited at Portuguese Institute of Oncology of Porto (IPO Porto). The *PTGS2* polymorphisms (-1195A>G, -765G>C and 8473T>C) and the *HPGD* polymorphisms (rs8752, rs2612656 and rs2555639) were characterized by allelic discrimination with Real-Time PCR. The -1195A>G polymorphism was associated to 2.04-fold increased predisposition to CRC onset. In a stratified analysis, men and ever-smokers carrying -1195GG genotype had an increased risk for CRC development (OR 3.10; 95%CI: 1.22-7.88; *P*=0.013 and OR 3.90; 95%CI: 1.17-12.96; *P*=0.019, respectively). The -765G>C polymorphism was associated with a protective trend for CC homozygous (OR 0.62; 95% CI 0.22-1.71; *P*=0.345). The rs2612656A>G polymorphism was significantly associated with a protective role for CRC (OR: 0.62; 95% CI: 0.43-0.90; *P*= 0.011) and a measurable interaction was detected between the AG genotype and gender (OR 0.38; 95% CI: 0.19–0.76; *P*=0.005 in females) or smoking habits (OR 0.53; 95% CI: 0.28–0.97; *P*=0.039 in never-smokers); more interestingly, the protective role seemed to be modulated by a high BMI also in individuals carrying the AG genotype (OR 0.39; 95% CI: 0.15–0.97; *P*=0.039 in individuals with BMI ≥30). For the rs8752C>T polymorphism, individuals carrying the CC genotype were associated with a nonsignificant 40% increase in risk of CRC (OR 1.41; 95% CI: 0.90-2.22; *P*=0.133) that reaches significance level when we assessed possible gene-environment interactions (OR 1.94; 95% CI: 1.04-3.61; *P*=0.035 in individuals over age 59). These findings revealed that -1195A>G and -765G>C *PTGS2* polymorphisms and rs2612656 and rs8752 *HPGH* polymorphisms appear to modulate the genetic susceptibility to CRC onset. This is the first study to evaluate the association between *HPGD* polymorphisms and CRC susceptibility in a Portuguese population. Our results support the importance of the PGE2 pathway in CR carcinogenesis showing that a better understanding on CRC

etiology might allow the characterization of sub-groups of individuals at higher risk who will most benefit from personalized chemopreventive strategies.

Keywords: Colorectal Cancer; *PTGS2*; *HPGD*; Polymorphism

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ABBREVIATIONS

3'-UTR - 3'- untranslated region

15-PGDH - 15-hydroxyprostaglandin dehydrogenase

APC – Adenomatous Polyposis Coli

AU - Adenylate-Uridylate

BMI – Body Mass Index

COX - Cyclooxygenase

COX-2 – Cyclooxygenase 2

COX-1 – Cyclooxygenase 1

CR - Colorectal

CRC – Colorectal Cancer

DNA – Desoxyribonucleic Acid

FAP - Familial Adenomatous Polyposis

FFPE – Formal Fixed Paraffin Embedded

FLAP – 5- Lipoxygenase Activating Protein

hMLH1 – Human Mut L Homolog 1

hMSH2 – Human Mut S Homolog 2

HNPCC - Hereditary Non Polyposis Colorectal Cancer

HPGD - Hydroxyprostaglandin Dehydrogenase

IARC - International Agency for Research on Cancer

IBD - Inflammatory Bowel Disease

IPO – Portuguese Institute of Oncology

KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

LTB4 – Leukotriene B4

mRNA – Messenger Ribonucleic Acid

NSAIDs - Non-Steroidal Anti-Inflammatory Drugs

OR – Odds Ratio

p53 – Protein 53

PG – Prostaglandin

PGD – Prostaglandin D

PGD2 – Prostaglandin D2

PGE – Prostaglandin E

PGE2 - Prostaglandin E2

PGF2 – Prostaglandin F2

PGI2 – Prostacyclin I2

PTGS - Prostaglandin Endoperoxide Synthase

PTGS2 - Prostaglandin Endoperoxide Synthase 2

RORENO - Registo Oncológico Regional do Norte

SNPs - Single Nucleotide Polymorphisms

Sp1 - Stimulatory protein 1

Tag-SNPs - Tagging Single Nucleotide Polymorphisms

TXA2 – Thromboxane A2

WHO – World Health Organization

INTRODUCTION

1. Cancer

Cancer is the leading cause of death in developed countries and has a profound impact on patients, family and society and is probably the most feared diseases in the population. In 2008, it was estimated that 12.6 million people were diagnosed with cancer across the world and 7.6 million cancer deaths occurred worldwide [1]. According to Bray *et al.* there will be almost 22.2 million new cases diagnosed annually worldwide by 2030 [2].

Cancer is a term used to describe a mass of cells that are abnormally growing without control and are able to invade other tissues. The carcinogenesis process is characterized by the evolution of normal cells into neoplastic cells by the acquisition of six biological capabilities: sustaining proliferative signaling, escape growth suppressors, cell death, angiogenesis, invasion and metastasis [3] - figure 1. Cancer is considering a multifactorial disease where both genetic and environmental factors are part of a multistep process [4].

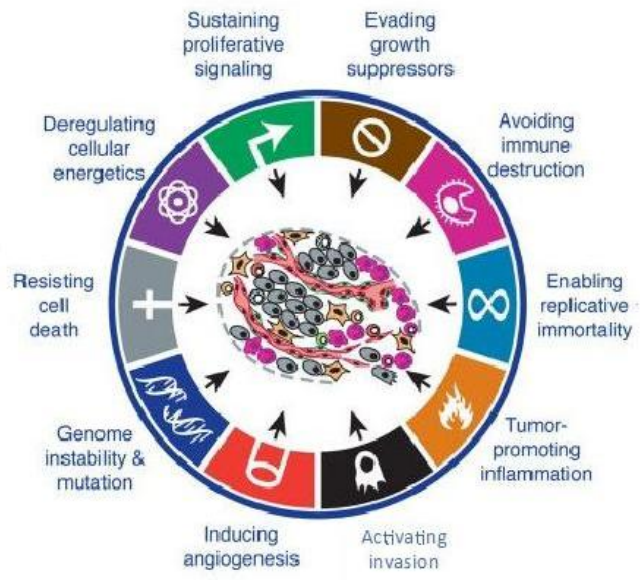

Figure 1. The Hallmarks of Cancer – six core and two emerging - and Enabling Characteristics (adapted from Hanahan and Weinberg, 2011) [3].

Of all carcinogenic agents, there are many, which are well known to increase the risk of cancer development including tobacco, radiation, lack of physical activity, obesity, environmental pollutants and infection agents [5]. These agents can directly damage genes or combine with existing genetic determinants leading to cell deregulation and the development of neoplastic cells [6].

1.1 Colorectal cancer epidemiology

Colorectal cancer (CRC) is a serious health problem with an annual incidence of 1.235,108 cases and a mortality rate of 609,051 cases per year [1]. It is the third most common malignancy and the fourth most common cancer cause of death worldwide, as can be seen in Figure 2.

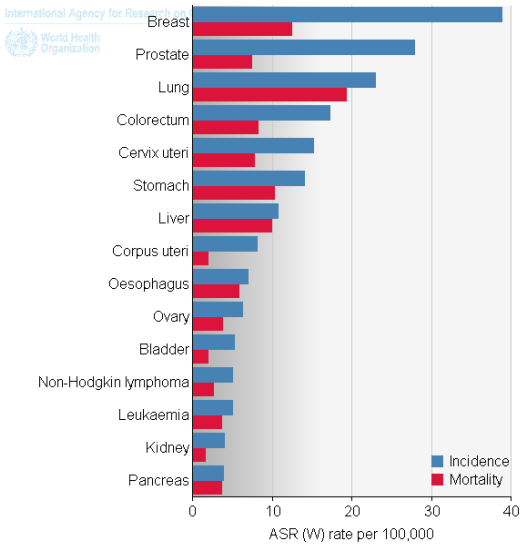

Figure 2. Estimated age-standardised incidence and mortality rates in both genders worldwide [1].

The worldwide distribution of CRC is very heterogeneous and it is more common in developed countries than in developing ones. In fact, United States of America, Canada, Western Europe, Australia, Japan and New Zealand are among the countries with highest incidence rates [1] - figure 3. According to the World Health Organization (WHO), CRC is the most common malignant tumor in the European Union [7] with about 4% of men and 3% of women to be at risk of developing CRC until age 75 [8].

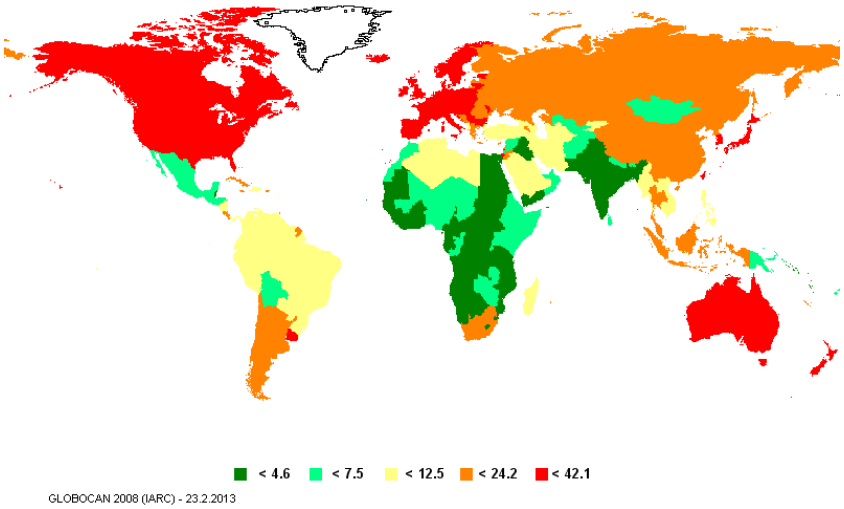

Figure 3. Incidence distribution of colorectal cancer worldwide in 2008 (per 100.000) [1].

In Portugal, CRC incidence is of 6,952 cases and mortality of 3,691 cases per year [1]. According to published data, mortality has significantly increased over the last few decades and still is the second most common cause of cancer-related deaths in women (after breast cancer) and in men (after lung cancer) [1]. According to *Registo Oncológico Regional do Norte* (RORENO) [9], the total number of new patients seen at the IPO in Oporto during the year of 2010 was 10241. Of these, 7050 (69%) were diagnosed with malignant tumors including 433 from colon and 396 from rectum.

Surviving CRC highly depends on the stage of the disease when it is diagnosed. Usually people with cancers that are detected at the localized stage had a 90% 5-year survival rate while only 10% of the people diagnosed for distant metastatic cancer has the chance of surviving [10,11]. In general, earlier stages diagnoses increase the chance of survival. Nevertheless, in Europe, CRC is the third leading cause of cancer death with 212,219 deaths in 2008 [1].

Hence, as CRC incidence is expected to increase in the near future as a result of population aging and increased life expectancy, some regions in Europe, where preventive strategies, early screening and treatment were adopted, are succeeding in reducing the number of cancer deaths [12-14].

1.2 CRC etiology and risk factors

In the vast majority of cases, CRC is sporadic (with no background of a family history of the disease), nevertheless there are genetic hereditary syndromes such as familial adenomatous polyposis (FAP), which accounts for approximately 1% of cases, and hereditary non polyposis colorectal cancer (HNPCC), which accounts for 5% to 10% of cases [15,16].

Sporadic CRC is considered to be an environmental disease since it is associated with lifestyle factors that contribute to increased risk of CRC. Some of the evidence of environmental risk factors comes from studies with migrants. The incidence rate of CRC in migrants tends to increased when they travel from low-risk to high-risk countries [17,18].

In fact, CRC is a multistep/multifactorial disease on which several risk factors are associated with its development. Generally, we may divide risk factors in two distinct categories: 1) modifiable factors (tobacco, lack of physical activity, obesity, etc.) and 2) non-modifiable factors (age, race, family history) [19,20].

Age is consistently considered a major risk factor for the development of sporadic CRCs since its incidence begins to rise between the age of 40 and 50 years. In fact, age-specific incidence rates increase significantly in each subsequent decade thereafter [19,20]. Other non-modifiable factors include Inflammatory Bowel Disease (IBD) [21], personal or family history of CRC or colorectal polyps [22], and a genetic syndrome such as FAP or HNPCC [23,24].

The International Agency for Research on Cancer (IARC) has been showing that there is sufficient evidence to conclude that tobacco is highly associated with CRC development [25]. Several studies have shown that carcinogens found in tobacco amplified cancer growth in the colon and rectum and the risk of being diagnosed with this type of cancer [26,27].

Other factor risk includes diet and physical activity. High levels of physical activity decrease the risk of colon cancer by almost 50% [28] and also contributes to decrease obesity, which is another factor associated with CRC [29]. Several studies have found that high consumption of red meat and low fruit and vegetables intake increases the risk of CRC [29-33]. Moreover, CRC has been linked to alcohol consumption. Individuals who consume less than one drink per day have lower risk of CCR than those who have a lifetime average of 2 to 4 alcoholic drinks per day [34].

Despite the importance of early detection, there are many studies suggesting that modifying lifestyle would reduce significantly CRC incidence [35-37].

1.3 CRC biopathology and carcinogenesis

CRC includes all neoplastic malignant lesions that are in the large bowel (cecum, ascending/transverse/descending colon and sigmoid), rectum and anal canal [38]. CRCs develop from apparently normal mucosa into a benign precursor stage, the premalignant polyp, and can progress to invasive disease [39]. The collaboration among surgeons, gastroenterologists, oncologists, pathologists, geneticists and molecular scientists allowed us to understand the dynamic molecular and histopathological changes that occur in CCR.

The development of CRC is often very silent, since in the majority of cases it is asymptomatic. Nevertheless, in symptomatic cases, there are some differences regarding the signs and symptoms depending on the localization of the tumors: in the ascending colon it is frequent to find anemia; while in the descending/sigmoid colon it is

frequent to find obstipation and hematochezia [40,41]. In fact these are the reasons why so frequently CRC is found in advanced stages. If diagnosed early, CRC has a cure rate of 90%, while if diagnosed in late stages, this percentage decreases up to 5% in metastatic cases [42]. Hence, the success rate is depending on the ability to detect early stage cases.

The advances in genetic and molecular biology of cancer led to a rapid knowledge of gastrointestinal cancers pathogenesis, nevertheless it took about a decade to demonstrate all the molecular mechanisms that allow the transformation from normal mucosa to adenocarcinoma [43] - figure 4. CRC carcinogenesis is known to develop according to a cascade named “adenoma-carcinoma sequence” [45]. This pathogenic pathway occurs through a number of morphologically identifiable stages: colon epithelial proliferation, small adenomas formation, gradual increase of adenomas and their degree and carcinoma. Several authors have described this pathway as an evolution of premalignant adenomatous polyps. There are evidences that support this theory: 1) Patients who have undergone resection of adenomatous polyps are at increased risk for subsequent development of CRC [46]; 2) Adenomatous polyps occur in younger persons than do carcinomas [47]; 3) Colonoscopic polypectomy reduces the expected incidence of CRC [48] and 4) Adenomas are present in more than 30% of persons older than 50 years and their prevalence increases with age [49].

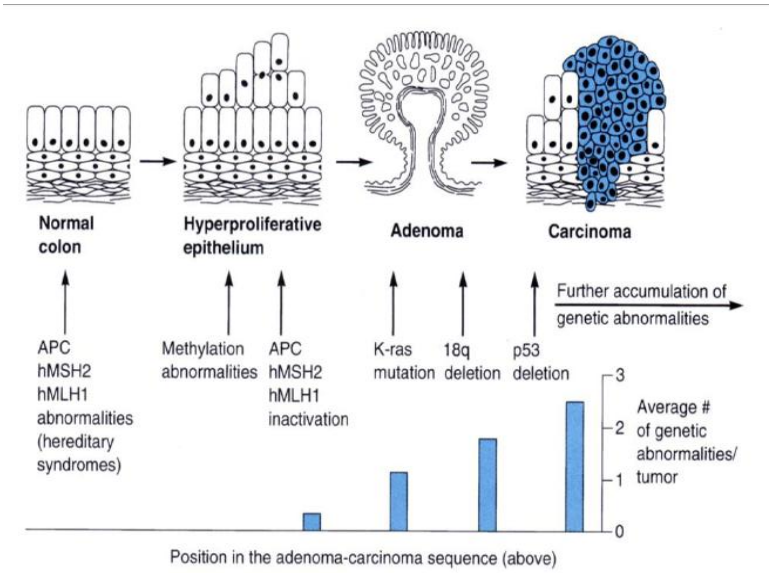

Figure 4. CRC carcinogenesis (Kutz H *et al.*, 2004) [44].

This cascade is a result of genetic mutations, along with environmental influences, causing inactivation or promotion of specific genes known as tumor suppressor and tumor promoter genes [50]. This “adenoma-carcinoma sequence” spans for over 10-15 years and therefore provides excellent opportunities for CRC prevention [45]. Early screening, treatment and follow-up of individuals previously diagnosed with adenomas (who have high risk of recurrence and develop cancer) is the keystone for CRC prevention and the best approach for CRC-associated mortality reduction. Thus, improved CRC screening guidelines and preventive strategies are still required to reduce the burden of this cancer.

2. Inflammation and CRC

One of the highlighted fields of interest in the development and progression of CRC has been inflammation, which is considered to have a crucial role in cancer initiation [51,52]. In fact, literature reports the evidence that CRC is more frequently found in patients with IBD [53]. And despite patients with IBD represent a small part of CRC cases (1–2%), these patients are among those at greatest risk. In addiction it has been described that patients with prolonged (>30 years) and extensive colitis, have an increased risk of CRC development [54]. It is also known that chronic inflammation promotes carcinogenesis by inducing gene mutations, inhibiting apoptosis, or stimulating angiogenesis and cell proliferation [55]. On the other side, many authors have been supporting that the use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence and mortality of several cancers, including CCR [56].

2.1 COX and NSAIDs

Cyclooxygenases (COXs), also known as prostaglandin endoperoxide synthases (PTGS), are rate-limiting enzymes that convert free arachidonic acid into several prostaglandins, namely prostaglandin E2 (PGE2) [57], inducing the immune response via the “inflammation pathway” [58] - figure 5.

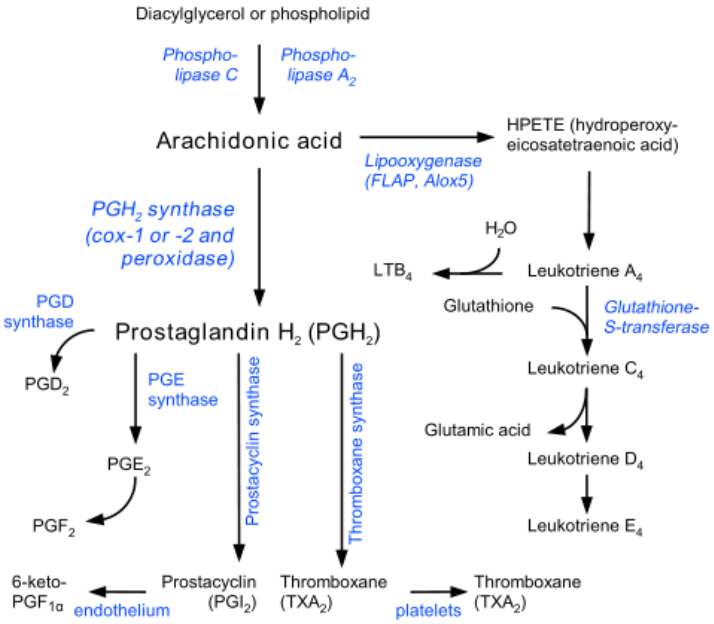

Figure 5. Arachidonic acid cascade pathway (http://en.wikipedia.org/) [59].

This enzyme has at least two isoforms identified usually referred as COX-1 and COX-2 [60]. COX-1 is constitutively expressed in a wide range of organs and responsible for tissue homeostasis; COX-2, is almost undetectable under normal physiological conditions but readily induced in response to tumor promoters, growth factors and inflammatory cytokines [60,61]. In fact, COX-2 was shown to be up-regulated in 40-50% of adenomas and 85% of CRC and is considered a key and early oncogenic event in colorectal carcinogenesis [60-62].

The most abundant PG in colorectal tumors is the COX-2-derived PGE2, which stimulates cell proliferation, invasiveness and migration, enhances angiogenesis, inhibits apoptosis and modulates immunosuppression [63]. PGE2 promotes carcinogenesis and cancer progression due to its interference with the hallmarks of cancer development, including inflammation [64]. The presence of PGE2 in the microenvironment seems to favor tumor progression but a better understanding of PGE2 synthesis pathway in colorectal carcinogenesis is necessary to help target potential measures of cancer prevention and to allow the identification of higher risk individuals. The steady-state level of PGE2 is maintained in the tumor microenvironment by a balance between biosynthesis and degradation. As PGE2 levels are important for tumor development, the ability to control its expression is necessary to suppress tumor growth. The 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is an enzyme, encoded by the hydroxyprostaglandin dehydrogenase (*HPGD*) gene that converts PGE2 into 15-keto-prostaglandin, eventually leading to the inactivation of PGE2 [64-67]. Therefore, 15-PGDH is considered to have tumor suppressor activity in human gastrointestinal malignancies by counteracting COX-2 actions [67,68]. In fact, 15-PGDH is normally expressed in gastrointestinal mucosa and has been shown to be down-regulated in CRC, thereby providing a potential mechanism for local accumulation of PGE2 and consequently favor tumor development [69]. These findings and the fact that PGDH expression is reduced in inflammatory bowel disease [70], a condition associated with markedly increased CRC risk, increase the oncogenic potential of the PGs synthesis pathway.

Therefore, to prevent CR carcinogenesis is important to control inflammation and many have suggested that the use of NSAIDs could benefit patients. NSAIDs are mainly responsible for the inactivation of COX enzymes [71], and clinical trials among either NSAIDs or selective COX-2 inhibitors have been revealing promissory results in colorectal adenoma prevention. Several studies suggest that the number of adenomas may decrease by almost 50% [72-77].

3. Genetic predisposition for CRC development

It is known that genetic predisposition has a key role in CRC development. An extensive analysis of twins from Sweden, Denmark, and Finland demonstrated that heritability contributes to 35% of all CRC cases [78]. In addition, almost 20% of all patients with CRC have a positive familial history [79] whereas only 5 to 10% fulfills the criteria for hereditary colorectal cancer, what could suggest the implication of low-penetrance alleles for the remaining familiarity [80]. Hence, there is a significant importance of low-penetration risk factors for the development of CRC.

Polymorphisms are common DNA genetic alterations present in at least 1% of the general population [81] that have been associated with differential susceptibility to the development of several diseases, including cancer. Single Nucleotide Polymorphisms (SNPs) are the simplest type of polymorphism and result from a single base mutation, which substitutes one nucleotide for another [82]. Since SNPs are inherited from one generation to the next, less mutable and have high frequency in the genome, they are largely used in the genetic dissection of diseases such as cancer [82].

Nowadays several epidemiological and molecular studies are focusing on SNPs and their ability to modulate the risk of various cancer and some of them have concluded that various gene polymorphisms can affect the risk of cancer development in almost all the populations around the world [83,84].

The number of studies attempting to determine which SNPs are likely to be implicated in CRC carcinogenesis, either alone or by interactions with environmental factors, continues to increase. Knowing that chronic inflammation is implicated in the etiology of CRC, our group has been developing molecular epidemiology studies on genes that are strongly related with inflammation, in particularly, genes that influence the levels of PGE2 in the tumor microenvironment, such as *PTGS2* and *HPGD*.

3.1 Genetic variability in *PTGS2* gene

COX-2 is an enzyme encoded by the *PTGS2* gene, which spans about 8.3 kb on chromosome 1q25.2-q25.3 and has 10 exons and 9 introns [85]. Transcriptional regulation has been shown to be a major mechanism in COX-2 expression [86]. The study of the *PTGS2* promoter region has recognized several potential transcription regulatory elements, which may play a crucial role in the regulation of *PTGS2* transcription [87,88]. In addition to variations in the promoter region, sites in the 3'-untranslated region (3'-UTR) of the gene may also be involved in CRC development.

This region is extremely important because it influences the mRNA transcripts 'stability and ultimately regulates prostaglandins production [89-91].

PTGS2 is a highly polymorphic gene, which contains more than 500 polymorphisms described so far. However, only a few have been associated with gastrointestinal tumors development [92-94].

3.1.1 The -1195A>G polymorphism

The -1195A>G polymorphism is located in the promoter region of *PTGS2*, characterized by a adenine (A) to guanine (G) transition at position -1195 from exon 1. According to Tang *et al.* [95] a significant variation was observed in the frequency of this polymorphism. The -1195A allele frequency in Asian populations is significantly lower (46%) than that in European (75%) and that in mixed ethnicity (82%) [95].

So far, functional studies have shown that the -1195A>G polymorphism regulates *PTGS2* transcription activity and COX-2 expression, by creating different recognition binding sites for nuclear proteins [96,97].

In a previous observational study developed by Zhang *et al.* [96] in a Chinese population, the -1195AA genotype was associated with increased risk of esophageal squamous cell carcinoma. In addition, the AA genotype was further associated with a higher genetic predisposition for the development of gastric and colorectal adenocarcinomas in Asiatic populations [98,99]. However, another case control study reported a decreased risk for CR adenoma onset associated with 1195AA genotype [100], which, although rather unexpected, was further supported by a study involving FAP patients reporting an increased risk association between -1195GG genotype and CRC onset [101]. Similarly, our group in a preliminary study demonstrated that individuals carrying at least one G allele had a higher genetic predisposition to developed CRC [102]. These contradictory associations seem to suggest that the behavior of this polymorphism can be modulated by other factors.

3.1.2 The -765G>C polymorphism

-765G>C is the most extensively studied polymorphism on *PTGS2*, especially in gastrointestinal cancers. It is located in the promoter region of *PTGS2*, characterized by a guanine (G) to cytosine (C) transition at position -765 (-765G>C) from exon 1 [103]. The -765C allele is quite frequent in Caucasians and African Americans, with

frequencies of 25-50% in different countries while the variant allele is fairly rare in Asians [104]. This polymorphism appears to disrupt the recognition binding site for *Stimulatory protein 1* (Sp1), which is considered to be a positive activator of transcription, leading to a 30% reduction of the *PTGS2* promoter activity *in vitro* [103]. In contrast, -765G>C also creates a binding element for the E2F transcription factor, a cyclin-dependent regulator of expression of several genes [96,98], that might eventually lead to an increased prostaglandin biosynthesis which in chronic conditions induces cancer development [104].

In Italy, it was observed that individuals carrying the -765C allele had lower risks of myocardial infarction and ischemic stroke [105], and in Spain it was associated with lower COX-2 expression and reduced atherosclerosis in patients with hypercholesterolemia [106]. In contrast, our group considered the -765C allele an increased susceptibility marker for gastric adenocarcinoma development in patients with atrophy or intestinal metaplasia [107].

Several studies have investigated the role of -765G>C polymorphism in CRC or CR adenomas onset, but their results have been inconsistent. One study found no association between this polymorphism and CRC [108] or CR adenomas [109]. Another study involving a population of Japanese men showed a decreased risk of CR adenomas linked with the GC genotype [100] while others associated this genotype with a significant increased risk of CRC [110]. Ulrich *et al.* related the CC genotype with decreased risk of CR adenomas among individuals who did not take aspirin or other NSAIDs [111]. More recently, it was observed an increased risk for CRC development in individuals carrying the GG genotype in a Dutch population [112].

3.1.3 The 8473T>C polymorphisms

The 8473T>C polymorphism is located in the 3'-UTR region of *PTGS2*, characterized by a thymine (T) to cytosine (C) transition at position 8473 (8473T>C). Several studies revealed that the 8473C allele frequency in Caucasians is approximately 30% [92,100,108]. The 3'-UTR region contains highly conserved adenosine and uridine rich elements, which are known as AU rich elements [89]. This motif is known to regulate mRNA stability and degradation of several other early intermediate genes encoding inflammatory mediators and also enhance the mRNA transcripts' stability leading to an increased prostaglandins production [113-117].

Some studies that investigated the *PTGS2* 8473T>C polymorphism, found the C variant allele to be associated with increased risk for non-small cell lung, breast and colorectal cancers [108,115,118]. In contrast to these results, other studies reported a protective effect of the same genetic polymorphism against lung and gastric cancers [92,119,120]. However, there are some studies that establish no association between this polymorphism and CRC cancer [102,121,122]. Regarding to CR adenomas, one study found the TC genotype to be associated with 47% increased risk [123] while other associated it with 31% increased risk [114]. In contrast, Gong and coworkers [109] reported evidence that individuals with the COX-2 8473C variant allele who also regularly take NSAIDs may be at low risk for CR adenomas.

These results may show the importance of study molecules involved in the arachidonic acid cascade and their implications in the etiology of several types of cancer.

3.2 Genetic variability in *HPGD* gene

15-PGDH is an enzyme encoded by the *HPGD* gene, which spans about 31 kb on chromosome 4q34-q35, contains 7 exons, 6 introns [124] and several regions within the 5-flanking region with clustered putative transcription factor-binding sites [125].

As well as *PTGS2*, *HPGD* is also a highly polymorphic gene with more than 700 polymorphisms described. However, only in the last few years some interest has been aroused in the role of genetic variations in *HPGD* gene. In 2008, recessive mutations in *HPGD* were identified as responsible for primary hypertrophic osteoarthropathy [126]. These mutations lead to a chronic increase in PGE2 levels, which is known to be implicated in colorectal cancer [127,128]. Besides that, it has been reported that this gene can act as tumor suppressor in different cancers, such as bladder, breast, gastric, lung and colorectal [129-138].

Only five studies have been conducted to evaluate the implication of polymorphisms in this gene in colorectal carcinogenesis [139-143]. Despite the lack of functional data describing polymorphisms in *HPGD* gene on development of CRC, we were able to selected three polymorphisms that were previously associated with colorectal lesions [139-141]. The rs8752T>C polymorphism is located in the 3'-UTR region of *HPGD*, characterized by a thymine (T) to cytosine (C) transition from exon 7. The rs2612656A>G polymorphism is characterized by a adenine (A) to guanine (G) transition in the intron between exons 4 and 5. Both SNPs were previously associated with a higher CRC risk [141]. Quite contrary to these results, Frank *et al.* [139] found rs8752 to be significantly associated with decreased CRC risk both among individuals

with a BMI<30 and among smokers. The rs2555639T>C is located in the 5' UTR region of *HPGD*, characterized by a thymine (T) to cytosine (C) transition. And a recent study suggests that the rs2555639 T allele is associated with an increased risk of colorectal cancer, and that carriers of this risk allele exhibit lower expression of 15-PGDH in the colon [140].

Since the information about *HPGD* polymorphisms is scarce and genotyping all the SNPs present in this gene were practically infeasible, a tag-SNPs approach was chosen in four studies [139,141-143]. With this approach it is possible to sequence only a small number of SNPs, called *tag SNPs*, and then infer the rest of SNPs (or certain suspicious SNPs) based on the sequenced tag SNPs. So, the rs8752T>C and rs2612656A>G polymorphisms represent a haplotype block. The identification and analysis of haplotypes is expected to play a key role in disease association studies [144].

AIM OF THE STUDY

It is plausible, that some genetic polymorphisms in PGE2 pathway genes might have functional repercussion on protein expression/function and expectably in PGE-2 levels contributing to colorectal carcinogenesis. However, more studies are needed to clarify the heterogeneity observed between published studies and to allow us the recognition of individuals at higher risk for CRC that may beneficiate from optimized preventive strategies.

The aim of this study was to evaluate the influence of the -1195A>G (rs689466), -765G>C (rs20417) and 8473T>C (rs5275) *PTGS2* polymorphisms and rs8752T>C, rs2612656A>G and rs2555639T>C *HPGD* polymorphisms on the development of CRC in a population from the Northern region of Portugal.

MATERIAL AND METHODS

1. Study design

We have designed a retrospective case-control hospital-based study gathering patients with CRC and healthy controls.

2. Study population

This study included 798 participants: 254 histologically confirmed CRC patients and 544 cancer-free controls, from the northern region of Portugal and recruited at the Portuguese Institute of Oncology of Porto (IPO Porto).

Written informed consent was obtained from all recruited participants before their inclusion in the study, according to the Declaration of Helsinki. This research project was approved by the Ethics Committee of the IPO Porto (ref. 0084/08) and *Comissão Nacional de Protecção de Dados* (ref. 6619/2011) that is the Portuguese Data Protection Authority.

2.1. Control Group

In this group, individuals between 50 and 75 years of age, without any clinical evidence of CRC or other oncologic malignancy were randomly recruited from the blood donor's service at IPO between July 2005 and February 2008.

2.2. CRC Patients Group

Patients with histopathologically confirmed CRC newly diagnosed between January 2002 and September 2007 were enrolled in this study. These patients were selected from a colonoscopy database from the Gastroenterology Department, aged 50–75 years, without previous history of inflammatory bowel disease or hereditary syndromes and whom were scheduled for a follow-up consult at *Serviço de Gastrenterologia* or *Unidade de Digestivos* at IPO Porto during March and May 2008 (n=387 cases).

Two-hundred and fifty four CRC patients were included out of the 387 expected to be recruited. During the recruitment or afterwards by telephone interview patients were asked to recall their lifestyle habits (smoking behavior, BMI, etc) in the previous year of CRC diagnosis. Medical records were reviewed to extract the clinicopathological variables (stage, tumor grade, presence of synchronous and metachronous lesions) and to exclude misclassification bias.

3. Sample collection and biological processing

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

For patients unable to provide a blood sample, the DNA was extracted from formalin fixed paraffin embedded (FFPE) blocks from the Pathology Department of our institute. Two to four 10µm thickness section were used in each extraction depending on the size of tissue area (1.5-3 cm^2). Briefly, the CRC tissue specimens from each glass slide were scraped, using a clean razor blade, into a 1,5-ml microcentrifuge tube. The samples were deparaffinised in xylene for 10 minutes, at room temperature, followed by centrifugation at 14.000g-16.000g for 3 minutes. The tissue pellets were then rehydrated with 1ml of absolute ethanol, followed by centrifugation at 14.000g-16.000g for 3 minutes and the supernatant was discarded. This step was repeated twice. Then, the tube was maintained open for 15 minutes to evaporate any remaining ethanol. Further steps of DNA isolation were performed using the GRS Genomic DNA Kit – Tissue, in accordance with the manufacturer's protocol (GRiSP, Porto, Portugal).

DNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at -20°C until genotype examination. The DNA quality was determined by measuring the optical density (OD) 260/280 ratio.

4. Validation of DNA genotyping extracted from FFPE samples

To assess whether DNA isolated from FFPE sections is reliable for retrospective genotyping we compared the genotypes of 20 somatic DNAs extracted from FFPE specimens to germline DNAs isolated from fresh peripheral blood from the same patients. The genotypes were highly concordant (100%).

5. Polymorphism selection and genotype characterization

As systematically reviewed by our group, there are some observational data supporting the involvement of *PTGS2* polymorphisms in gastrointestinal tumors development [13]. The *PTGS2* polymorphisms included in this study (rs20417, rs689466 and rs5275) were selected based on: (1) previous evidence of association with colorectal tumor risk; (2) biological plausibility; and (3) minor allele frequency (MAF) of at least 15%.

HPGD polymorphisms where select by literature review of previous association with colorectal tumors onset (rs8752, rs2612656 and rs2555639).

All polymorphisms were characterized through allelic discrimination (Real-Time Polymerase Chain Reaction) using validated TaqMan® SNP genotyping assays, with the exception of the polymorphism -765G>C (rs20417) which was custom designed (Applied Biosystems, Foster City, California USA) [Table I]. Reactions were performed on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, California USA) with a 6µl final volume mixture containing 2,5µl of the 2x TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, California USA), 2,25 µL TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) and 20ng of genomic DNA. Thermal cycling conditions were: 95°C for 10 minutes, followed by 40 cycles that consisted of denaturation at 92°C for 15 seconds, annealing and primer extension at 60°C for 1 minute. The post-read was performed at 60ºC for 1 min. Allelic discrimination was performed by measuring end-point fluorescence using ABI PRISM® Sequence Detection System (Applied Biosystems, Foster City, California USA).

Table I – Identification of the real-time genotyping assays, call rates and concordance rates for each SNP.

Gene	SNP ID (dbSNP)	Assay ID
COX2	rs689466	C___2517145_20
	rs20417	Custom assay
	rs5275	C___7550203_10
HPGD	rs8752	C___8848783_10
	rs2612656	C___15909858_20
	rs2555639	C___16038735_10

Cases and controls were genotyped randomly and results were independently analyzed by two researchers. For quality control: (1) blank templates were included in each 96-well plate to ensure contamination-free results; (2) the genotype interpretation was performed by two researchers independently; (3) and ten percent of all samples were randomly selected and re-submitted to a new genetic characterization to confirm the genotypes.

6. Statistical analysis

For genetic distribution analysis, the Hardy–Weinberg equilibrium was tested by the Pearson's goodness-of-fit test to compare the observed versus the expected genotype frequencies.

Data analysis was performed using the computer software IBM Statistical Package for Social Sciences-SPSS (IBM Corp., Armonk, New York, USA) for Macintosh (version 19.0). Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between the genetic variants and the risk for the development of CRC. Homozygotes for the allele with the highest frequency were used as the reference group for each OR estimation. The potential confounding variables such as age, gender, body mass index (BMI), and smoking habits were addressed either by being included as covariates in the multivariate analysis and/or through data stratification.

RESULTS

1. Participants' description

In total, 254 cases of colorectal cancer and 544 cancer free controls were included in this study. The characteristics of the study population are presented in Table II. The median age of cases and controls were 57 (50-69) and 63 (50-78) years, respectively. Males were overrepresented in both cases and in the controls category (60.2% vs. 64.2% in cases and controls, *P*=0.286) and over 75% of participants were overweight in either group (*P*=0.893). The majority of the participants had never smoked in both groups (61.8% in cases vs. 60.3% in controls, *P*=0.747).

Table II – Description of participants

	Cases (n=254)	Controls (n=544)	*P* value
Demographics			
Age (years)			
Mean ± sd	63±7.22	58±4.89	
Median(min-max)	63 (50-78)	57 (50-69)	<0.001*
Sex, *n* (%)			
Male	153 (60.2)	349 (64.2)	0.286
Female	101 (39.8)	195 (35.8)	
Lifestyle behaviors[a]			
BMI			
Mean (SD)	28±4.13	28±3.58	0.764*
Median (min-max)	28 (20-43)	27 (20-41)	
BMI category, *n* (%)[b]			
<25	36 (23.4)	58 (24.0)	0.893
≥ 25	118 (76.6)	184 (76.0)	
<30	114 (74.0)	184 (76.0)	0.652
≥30	40 (26)	58 (24)	
Smoking status, *n* (%)			
Never-smokers	97 (61.8)	240 (60.3)	0.747
Ever-smokers*	60 (38.2)	158 (39.7)	
Tumor characteristics			
Tumor location, *n* (%)			
Rectum	132 (52.0)		
Colon	121 (47.6)		
Stage, *n* (%)			
I-II	126 (49.6)		
>II	115 (45.3)		
Synchronous tumors, *n* (%)			
Yes	14 (5.5)		
No	224 (88.2)		
Metachronous tumor, *n* (%)			
Yes	3 (1.2)		
No	103 (40.6)		

BMI, body mass index.
[a]The numbers may not add up, as we were unable to gather this information for all participants, namely in the control group.
[b]Categorization based on the cutoff defined by WHO for overweight people [7]
[c]Smokers and ex-smokers were included
*P value was estimated using the non-parametric Mann–Whitney test

2. Genotype frequencies and risk estimates for *PTGS2* polymorphisms

The distributions of each *PTGS2* polymorphisms genotypes are shown in Table III. The genotypic distribution of all three SNPs in the control group was in agreement with the Hardy–Weinberg equilibrium principles (*P*≥0.05).

Regarding the -1195A>G *PTGS2* polymorphism, in the 252 analyzed cases, we observed that 58.7% were homozygous for A allele, 35.3% were heterozygous and 6.0% were GG homozygous while in the control group the genotype frequencies were: 68.3%, 28.3% and 3.4%, respectively. The GG and AG genotypes of the -1195A>G polymorphism were overrepresented in the group of cases leading to an increased risk for CRC, especially GG homozygous with 2-fold increased risk (OR 2.04; 95% CI: 1.00-4.15; *P*=0.046).

Regarding the -765G>C *PTGS2* polymorphism, in the 249 analyzed cases, we observed that 73.9% were homozygous for G allele, 60.0% were heterozygous and 2.1% were CC homozygous while in the control group the genotype frequencies were: 67.7%, 29.5% and 2.8%, respectively. Although the results for the development of CRC were not statistically significant, we observed a protective role trend for CC homozygous (OR 0.62; 95% CI: 0.22-1.71; *P*=0.345).

Regarding the 8473T>C *PTGS2* polymorphism, in the 250 analyzed cases, we observed that 38.4% were heterozygous while in the control group the genotype frequency was 42.3%. The TT genotype has a frequency of 49.6% in cases versus 48.9% in controls and the CC genotype was also more common in cases than in control group (12.0% vs. 8.7%, *P*=0.276). No significant differences in genotype distribution were noticed in this polymorphism.

Table III – Genotype frequencies among cases and controls and univariate OR (95% CI) estimation on the role of *PTGS2* polymorphisms in colorectal cancer onset

Polymorphisms	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	HWE
-1195A>G (rs689466)						0.979
AA	148 (58.7)	362 (68.3)	1.00	Reference	-	
AG	89 (35.3)	150 (28.3)	**1.45**	**1.05-2.01**	**0.024**	
GG	15 (6.0)	18 (3.4)	**2.04**	**1.00-4.15**	**0.046**	
-765G>C (rs20417)						0.972
GG	184 (73.9)	362 (67.7)	1.00	Reference	-	
GC	60 (24.1)	158 (29.5)	0.75	0.53-1.06	0.106	
CC	5 (2.0)	15 (2.8)	0.62	0.22-1.71	0.345	
8473T>C (rs5275)						0.976
TT	124 (49.6)	252 (48.9)	1.00	Reference	-	
TC	96 (38.4)	218 (42.3)	0.90	0.65-1.24	0.518	
CC	30 (12.0)	45 (8.7)	1.33	0.80-2.20	0.276	

OR, odds ratio; CI, confidence interval; HWE, Hardy- Weinberg equilibrium

3. Genotype frequencies and risk estimates for *HPGD* polymorphisms

Table IV shows the results for the three analyzed SNPs in *HPGD* gene. All genotypic distributions in the control group were in agreement with the Hardy-Weinberg equilibrium principles ($P \geq 0.05$).

Regarding the rs2555639T>C polymorphism genetic variation, in the 251 participants analyzed, the TT genotype had very similar frequencies in either groups (45.8% and 46.1% in controls and cases, respectively). Similarly, the heterozygous TC as well as the homozygous CC, also presented similar distribution between cases and controls (42.2% and 12.0% vs. 43.5% and 10.4%, respectively). There was no significant difference in the genotype frequencies between the group of cases and controls.

Regarding the rs2612656A>G *HPGH* polymorphism, in the 215 analyzed cases, we observed that 73.2% were homozygous for A allele, 21.1 % were heterozygous and 5.7% were GG homozygous while in the control group the genotype frequencies were: 65.4%, 38.4% and 4.2%, respectively. This polymorphism was significantly associated with a protective role for CRC (OR 0.62; 95% CI: 0.43-0.90, $P = 0.011$).

For the rs8752T>C *HPGD* polymorphism, in the 209 analyzed cases, we observed that 37.3% were homozygous for T allele, 45.6% were heterozygous and 17.1% were CC homozygous while in the control group the genotype frequencies were: 40.4%, 46.4% and 13.1%, respectively. Individuals carrying the CC genotype were associated with a nonsignificant 40% increase in risk of CRC (OR 1.41; 95% CI: 0.90-2.22; $P=0.133$).

Table IV – Genotype frequencies among cases and controls and univariate OR (95% CI) estimation on the role of *HPGD* polymorphisms in colorectal cancer onset

Polymorphisms	Cases n (%)	Controls n (%)	OR	95% CI	*P* value	HWE
rs2555639T>C						0.995
TT	115 (45.8)	243 (46.1)	1.00	Reference	-	
TC	106 (42.2)	229 (43.5)	0.98	0.71-1.35	0.892	
CC	30 (12.0)	55 (10.4)	1.15	0.70-1.90	0.575	
rs2612656A>G						0.899
AA	167 (73.2)	329 (65.4)	1.00	Reference	-	
AG	48 (21.1)	153 (38.4)	**0.62**	**0.43-0.90**	**0.011**	
GG	13 (5.7)	21 (4.2)	1.22	0.60-2.50	0.587	
rs8752T>C						0.996
TT	94 (37.3)	216 (40.4)	1.00	Reference	-	
TC	115 (45.6)	248 (46.4)	1.08	0.77-1.45	0.704	
CC	43 (17.1)	70 (13.1)	1.41	0.90-2.22	0.133	

OR, odds ratio; CI, confidence interval; HWE, Hardy- Weinberg equilibrium

4. Genotype-environment interactions

Tables V and VI show the results for the influence of *PTGS2* and *HPGD* polymorphisms in colorectal cancer stratified for age, sex, BMI and smoking status. Genotype association was tested for all heritability models and the most relevant will be presented.

Upon a stratified analysis we observed that males carriers the -1195GG genotype had a 3-fold increased risk for CRC onset (OR 3.10; 95%CI: 1.22-7.88; *P*=0.013). The enhanced susceptibility was even more noticeable for individuals who ever-smoked prior to their diagnosis (OR 3.90; 95%CI: 1.17-12.96; *P*=0.019).

A measurable interaction was detected between the rs2612656AG genotype and gender (OR 0.38; 95% CI: 0.19–0.76; *P*=0.005 in females) or smoking habits (OR 0.53; 95% CI: 0.28–0.97; *P*=0.039 in never-smokers). More interestingly, the protective role for CRC seemed to be modulated by a high BMI (OR 0.39; 95% CI: 0.15–0.97; *P*=0.039 in individuals with BMI ≥30).

The rs8752CC genotype was significantly associated with an increased risk in older individuals (OR 1.94; 95% CI: 1.04-3.61; *P*=0.035 in individuals over age 59).

Table V – *Odds Ratio* (95% CI) for the influence of *PTGS2* polymorphisms in colorectal cancer stratified for age, sex, BMI and smoking status

	-1195A>G (GG vs. AA)				-765G>C (CC vs. GG)				8473T>C (CC vs. TT)			
Stratification	N	OR	95% CI	*P* value	N	OR	95% CI	*P* value	N	OR	95% CI	*P* value
Age (years)[a]												
<59	271	1.66	0.50-5.50	0.297	268	0.36	0.05-2.83	0.275	214	1.74	0.78-3.85	0.172
≥59	270	2.18	0.85-5.62	0.099	295	0.95	0.25-3.60	0.605	235	1.12	0.57-2.22	0.738
Sex												
Male	341	**3.10**	**1.22-7.88**	**0.013**	357	0.67	0.18-2.51	0.398	295	1.21	0.64-2.28	0.556
Female	202	1.10	0.36-3.43	0.866	210	0.55	0.11-2.70	0.361	157	1.66	0.69-3.98	0.252
BMI (kg/m^2)												
<25	68	1.75	0.11-29.27	0.604	61	1.38	0.18-10.46	0.574	53	1.24	0.30-5.11	0.516
≥25	202	2.12	0.63-7.21	0.219	212	0.37	0.04-3.40	0.341	158	1.11	0.47-2.64	0.806
<30	200	3.19	0.90-11-28	0.060	194	1.20	0.26-5.60	0.553	160	0.96	0.40-2.26	0.321
≥ 30	70	0.62	0.51-0.75	0.392	74	0.54	0.44-0.67	0.304	51	1.84	0.43-7.85	0.321
Smoking status												
Never-smokers	227	0.59	0.12-2.83	0.302	238	0.39	0.05-3.31	0.338	190	1.30	0.52-3.25	0.568
Ever-smokers[b]	159	**3.90**	**1.17-12.96**	**0.019**	151	1.01	0.20-5.78	0.612	119	1.51	0.57-4.00	0.400

OR, odds ratio; CI, confidence interval; [a]Categorization defined by the overall median age; [b] Includes ever-smokers and ex-smoker

Table VI – *Odds Ratio* (95% CI) for the influence of *HPGD* polymorphisms in colorectal cancer stratified for age, sex, BMI and smoking status

	rs2555639T>C (CC vs. TT)				rs2612656A>G (AG vs. AA)				rs8752T>C (CC vs. TT)			
Stratification	N	OR	95% CI	*P* value	N	OR	95% CI	*P* value	N	OR	95% CI	*P* value
Age (years)[a]												
<59	212	1.27	0.53-3.05	0.591	346	0.63	0.36-1.13	0.119	200	1.10	0.52-2.23	0.816
≥59	230	0.94	0.50-1.76	0.847	347	0.69	0.41-1.15	0.149	220	**1.94**	**1.04-3.61**	**0.035**
Sex												
Male	281	0.86	0.44-1.65	0.642	137	0.79	0.50-1.23	0.289	268	1.27	0.71-2.28	0.416
Female	162	1.79	0.82-3.91	0.145	242	**0.38**	**0.19-0.76**	**0.005**	155	1.63	0.80-3.34	0.178
BMI (kg/m^2)												
<25	43	0.67	0.17-2.67	0.413	84	0.87	0.31-2.47	0.793	48	1.22	0.27-5.59	0.548
≥25	171	1.73	0.79-3.96	0.119	85	0.67	039-1.15	0.146	163	1.26	0.63-2.51	0.515
<30	152	1.89	0.48-7.40	0.356	268	0.88	0.51-1.52	0.640	157	1.15	0.55-2.40	0.714
≥ 30	62	1.89	0.48-7.40	0.356	85	**0.39**	**0.15-1.02**	**0.051**	54	1.33	0.40-4.40	0.636
Smoking status												
Never-smokers	181	1.66	0.76-3.61	0.203	296	**0.53**	**0.28-0.97**	**0.039**	192	1.27	0.62-2.60	0.506
Ever-smokers[b]	117	1.23	0.45-3.32	0.685	199	1.29	0.67-2.49	0.448	111	1.45	0.58-3.60	0.423

OR, odds ratio; CI, confidence interval; [a]Categorization defined by the overall median age; [b] Includes ever-smokers and ex-smokers

DISCUSSION

CRC incidence and mortality rate have decreased during the past few decades. Nevertheless it is still the fourth cause of cancer death worldwide and a major worldwide health problem especially in developed countries [1,145]. It has been shown that environmental and genetic factors have a substantial role in colorectal carcinogenesis and current research points that the identification of specific tumor promoter or suppressor genes will provide new targets for prevention, diagnosis and cancer therapies which may help to stop CRC burden and mortality [83,92].

Several studies have been confirming that inflammation plays a key role in the development of cancer, especially in CRC [51,52,56,107]. Inflammation is regulated by several cellular mechanisms that are used to control cellular abnormalities assumed as crucial for cancer prevention. In the inflammation process, *PTGS2* is a significant marker and its association with cancer is being widely studied [92,93,95]. Moreover, *HPGD* is considered an important regulator of inflammation and some studies point its importance in cancer [67,68].

COX-2 derived PGE2 is produced during the course of inflammation and is presence in tumor environment seems to favor tumor progression [64]. The steady-state level of PGE-2 is maintained in the tumor microenvironment by a balance between biosynthesis (by COX-2) and degradation (by 15-PGDH). Enhanced expression of COX-2 has been observed in many cancers, including CRC [146] and it is known that HPGD expression is lost in human colon cancer cells [69]. These findings indicate that genetic variants involved in the PG synthesis pathway may modulate the risk for CRC.

Firstly, it is important to characterize the limitations of this study to avoid misunderstandings of data. In this study we have included a total of 254 patients and 544 controls, nevertheless we were not able to obtain some clinical information from patients (such as weight, length and smoking habits). Therefore the analysis of BMI and smoking status may be biased due to the lack of some data and might explain the low statistical power found in some groups. Secondly, although we imposed the same age-restriction criteria in both groups, we noticed that CRC patients were significantly older than controls, which had impact on the stratified analysis.

Another limitation was the fact that not all patients were able to provide a blood sample, and DNA was extracted from FFPE. FFPE is the standard method for long-term preservation of clinical specimens and the usual technique for preserving specimens in hospital pathology departments. In recent years, due to the worldwide abundance of these samples, an increasing number of studies have been conducted using FFPE

tissue for nucleic acid extraction. However, the fixation process and storage of FFPE samples normally leads to nucleic acid degradation resulting in poor performance in genotyping studies. To overcome this we characterized all the SNPs using Real Time PCR that produce smaller size amplicons increasing the chance to detect a specific locus. We also validated the use of FFPE samples in this study by successfully demonstrating good concordance between genotypes obtained from DNA extracted from peripheral blood and FFPE samples.

Role of polymorphisms in PTGS2 gene on CRC onset

A number of associations between several SNPs in *PTGS2* and CRC have been reported [92,101,107,112]. Previously, in a preliminary study from our group it was reported that individuals' carriers of the -1195G allele were at an increased risk for CRC development [102]. In fact, the present study supports that first observation and results showed an over representation of AG and GG genotypes in the group of cases leading to an increased risk for CRC, especially GG homozygous with 2-fold increased risk. Moreover, a published study involving FAP patients also reported an increased risk association between -1195GG genotype and CRC onset [100]. However, these associations have raised some controversy. According to Zhang *et al.* [96] the -1195AA genotype was associated with a significantly higher risk for esophageal cancer. In addition, it was reported in several studies in Asiatic populations the association of the AA genotype with higher genetic predisposition for the development of gastric and CRC [97-99]. *In vitro* studies were developed to clarify these conflicting associations and while it was shown that the presence of -1195A allele creates a c-MYB-binding site, resulting in higher transcriptional activity of the *PTGS2* in esophageal cancer cells [96], Pereira *et al.* [97] using colon cancer cell lines reported that the -1195A to G substitution also creates an E-box motif which may lead to increased *PTGS2* transcription. Same results were observed in human hepatoma cell lines [147]. These two studies contribute for the support of our results corroborating the higher susceptibility for CRC that we found in individuals with GG genotype. Moreover, when we assessed possible gene–environment interactions, we observed a positive association between -1195GG genotype and CRC in males and in ever-smokers. Several studies already reported that CRC occurs earlier in smokers [148-151] and a meta-analysis study revealed that cigarette smoking doubles the risk of developing adenomatous polyps, known precursors of CRC [152]. Several theories regarding the smoke-induced cancer were developed and some authors suggest that one of the pathways could be due to COX-2 overexpression leading to a sustained inflammatory

activity [153,154]. Nevertheless however the pathogenesis of smoking related CRC is still lacking more studies and evidences.

Regarding -765G>C polymorphism we observed a trend for a protective role in individuals carrying the CC genotype, however these results were not statistically significant. It has been described that -765C allele has significantly lower promoter activity when compared with the -765G allele and therefore is associated with COX-2 under-expression [103]. Furthermore, in a previous study, the -765CC genotype showed a protective role for colorectal adenomas and hyperplastic polyps [111]. In addition, this genotype was also associated with a reduced risk for developing Crohn's disease in a Dutch population [155]. However, a recent meta-analysis found a significant association between the -765CC genotype and CRC risk in Asiatic populations [156]. The dual and antagonistic results observed with this polymorphism might have a biological interpretation, as the presence of allele C in *PTGS2* promoter region eliminates the recognition binding site for the Sp1 positive transcription factor leading to a 30% reduction of promoter activity in vitro [103] but it also creates a E2F homology binding site region that could explain the overexpression and increased risk [95,98]. Studies also suggested that ethnic and environment differences may influence the function of -765G>C polymorphism, therefore studies that investigate the association of specific life-style conditions with this polymorphism in patients with CRC may help to elucidate the contradictory results obtained with this polymorphism.

The 8473T>C *PTGS2* polymorphism is located within the functional region of the 3'-UTR of *PTGS2* which contains multiple elements that control mRNA stability and translation efficiency [113-115]. The functional importance of 8473T>C *PTGS2* polymorphism was recently investigated and an *in vitro* study showed that the presence of the variant C disrupted microRNA-mRNA interaction allowing COX-2 overexpression in CRC cells [157]. A recent metaanalysis has shown that the 8473C allele has been associated with a 1.25-fold increased susceptibility for colorectal adenoma and in contradiction with a protective role for gastric cancer [92]. However, there are some studies that establish no association between this polymorphism and CRC cancer [102,121,122]. In our study, the statistical analysis revealed no association between any of the genotypes of this polymorphism and CRC risk, which is in agreement with previous published studies [102,121,122]. So, this polymorphism may be a genetic risk factor in some populations but not in our.

Role of polymorphisms in HPGD gene on CRC onset

Regarding to HPGD, only more recently it has aroused great interest with few studies reporting an influence of HPGD SNPs in CRC [139-141]. To the best of our knowledge, this is the first study to evaluate the association between HPGD polymorphisms and CRC susceptibility in a Portuguese population.

Statistically analysis revealed that only the rs2612656AG genotype is significantly associated with protection for CRC development. These results are contrary to those obtained by Hoef *et al.* [141] where this variant was associated with higher CRC risk. First it is important to refer that differences in genetic ancestry may explain these inconsistent results since our samples were exclusively from the north region of Portugal whereas Hoef *et al.* [141] analyzed cases from ten European countries with different rates of CRC incidence. The diversity in genetic and environment backgrounds that is expected to find in the studied populations may also clarify these findings. When we assessed possible gene–environment interactions, a protection for CRC was observed for females and never-smokers carriers of the heterozygous genotype (AG). Smoking is associated with a higher risk of several types of cancer including CRC [148-152]. Recent metaanalyses suggest that current or former smokers had almost 20% increased risk of CRC when compared with never-smokers [158,159]. Moreover, it is known that men have a higher prevalence of colon polyps and tumors than women [160]. However, females smokers were found to be more susceptible to colon cancer than male smokers among Norwegians individuals [161]. These findings seem to suggest that other life-style factors, besides smoking, may interfere in the development of CRC. More interesting, our results demonstrated that heterozygous (AG) individuals with a BMI ≥ 30, despite borderline significance, seem to have a decreased risk for CRC. These results were unexpected since there is clear evidence that people who are obese (with BMI ≥ 30 kg/m2) have an increased risk for developing CRC [162,163]. Since the obese state leads to production of some molecules that are known to be involved in inflammation, such as prostaglandins, it is possible that some common mechanism between obesity and CRC exists [164,165]. Studies identifying genetic and biological determinants of obesity and its interactions with *HPGD* may help elucidate these results.

Regarding to rs8752 polymorphism, we found a nonsignificant 40% increased risk with the CC genotype that reaches significance level when we assessed possible gene-environment interactions. We observed an almost 2-fold increased risk in older individuals. Previous studies assessing the influence of rs8752 polymorphism on CRC risk reported opposite results: one found this polymorphism to be associated with CRC

risk [141] while other reported a protective role for CRC [139]. These variable findings and the increase risk that we observed in older individuals may be due to gene-gene and gene-environment interactions, which support the idea that CRC is a multifactorial disease.

Moreover and although a recent study has shown an association of rs2555639 T allele with increased risk of colorectal cancer [140], we were not able to replicate this result in our study.

As already mentioned, the heterogeneity observed among epidemiological studies might be explained by population stratification, also involving differences in genetic lineage, and by different environmental backgrounds, thus reinforcing the idea that it is important to evaluate the influence of SNPs in CRC in independent populations.

CONCLUSION and FUTURE STUDIES

With this study we attempted to replicate genetic associations between six polymorphisms (rs689466, rs20417 and rs5275 in PTGS2 and rs8752, rs2612656 and rs2555639 in HPGD) and CRC risk. All this polymorphisms were previously reported to have some association with CRC. Nevertheless, this is the first study to combine all genetic inflammation in one study.

Regarding to *PTGS2*, we found -1195GG genotype to be significantly associated with a increased CRC risk especially both among males and among ever-smokers. We also observed a protective role trend for individuals carrying the -765CC genotype.

Regarding to HPGD, we found rs2612656AG genotype to be significantly associated with a decreased risk especially both among females and never-smokers. We also observed that individuals with a BMI ≥ 30 presenting this genotype also seem to have a decreased risk for CRC. Regarding to rs8752T>C polymorphism, we found a nonsignificant 40% increased risk with the CC genotype and an almost 2-fold significant increased risk in older individuals carrying the same genotype.

In the future it will be interesting to characterize functionally the interaction between -1195A>G polymorphism and smoke-induced colorectal carcinogenesis to evaluate if this association may represent a risk model for CRC development. Also it would be appealing to examine a possible interaction between these polymorphisms and NSAIDs use that could allow us to define a pharmacogenomic profile. Furthermore, expression studies of rs8752T>C and rs2612656A>G *HPGD* polymorphisms may help elucidate their impact in CR carcinogenesis observed in our case-control study.

Therefore, the analysis of potentially functional SNPs in candidate genes might allow the characterization of sub-groups of individuals at higher risk for cancer development. These groups could have a more premature diagnosis and benefit from personalized chemopreventive strategies. Our data underlines the oncogenic importance of the PG pathway and its influence in the development of CRC cancer.

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