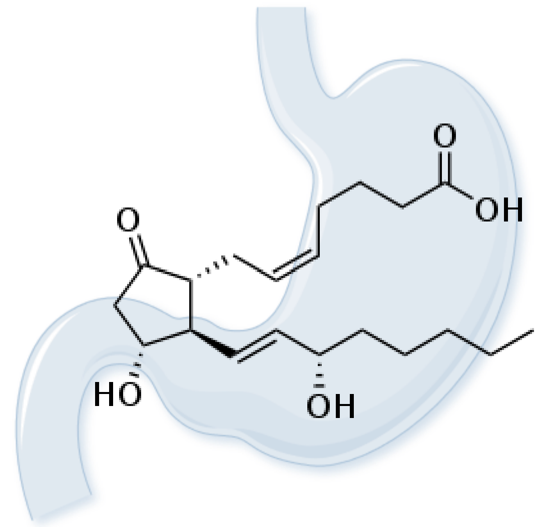
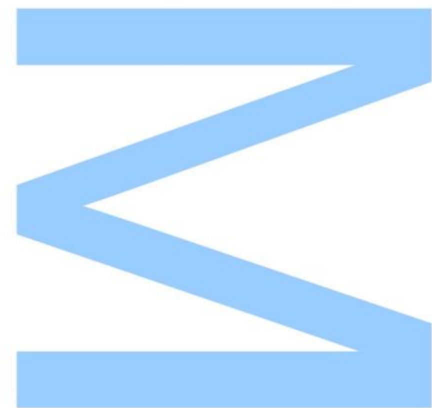


Genomic Characterization of the Prostaglandin E_2 Pathway in Gastric Cancer Development: An Opportunity for Personalized Screening?

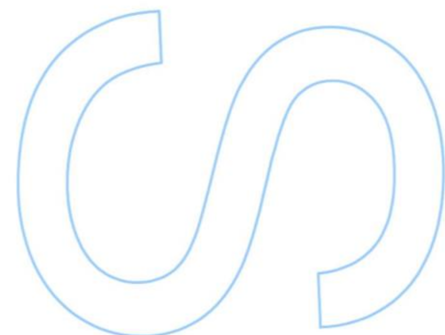


Catarina Filipa Pereira Lopes

Mestrado em Bioquímica

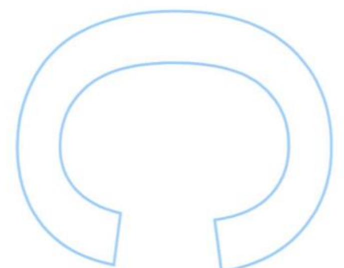
Faculdade de Ciências da Universidade do Porto (FCUP)
Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS)

2019



Orientador
Ana Carina Pereira, PhD, IPO-Porto

Coorientador
Ricardo Marcos-Pinto, MD, PhD, ICBAS



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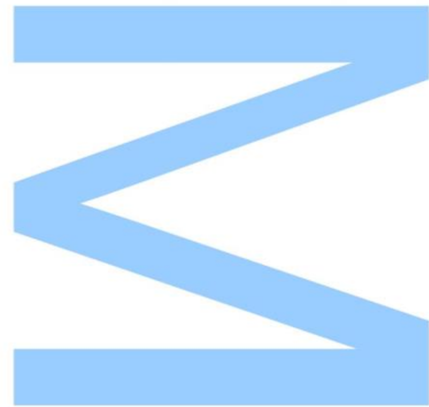
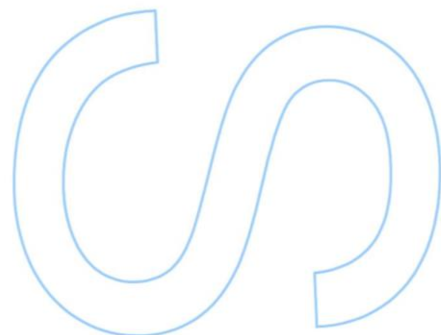
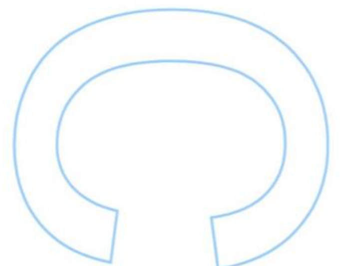
O Presidente do Júri,

Porto, ______/______/_________







Agradecimentos

Com o fim de mais uma etapa, não posso deixar de agradecer a todos os que contribuíram para este trabalho e que o tornaram possível.

Primeiro, à Doutora Carina Pereira, que me orientou durante esta tese e se mostrou incansável para que fosse concluída. Por todo o apoio, pelas horas sem dormir, pela resiliência perante os obstáculos que nos foram surgindo pela frente e pela amizade. O meu mais sincero obrigada.

Ao Prof. Doutor Ricardo Marcos-Pinto, por me ter aceitado como co-orientanda e por ter acreditado neste projeto. Obrigada por toda a cooperação.

Ao Prof. Doutor Rui Medeiros, líder do grupo de onde fiz parte durante este ano, por me acolher sem pensar duas vezes, por todo o encorajamento e por sempre estar disponível para ouvir as nossas propostas.

Ao Prof. Doutor Mário Dinis-Ribeiro pelo conhecimento transmitido e por potenciar a realização deste trabalho.

Ao Prof. Doutor Rui Henrique, atual Presidente do IPO-Porto, por colaborar neste estudo e facilitar o acesso às amostras biológicas arquivadas no Serviço de Anatomia Patológica desta instituição, que foram imprescindíveis.

Um especial agradecimento à Doutora Mónica Farinha, do Serviço de Anatomia Patológica do IPO-Porto, por toda a disponibilidade para selecionar e rever todos os cortes histológicos, apesar da carga de trabalho. À técnica Fernanda Silva e ao técnico Jorge Ferreira por todas as horas dedicadas ao corte dos blocos histológicos.

Ao Doutor Luís Lima, do Grupo de Patologia e Terapêutica Experimental, pelos conhecimentos transmitidos e pelo apoio na análise estatística dos resultados.

Ao Prof. Doutor Paulo Correia de Sá, diretor do Mestrado em Bioquímica, por me ter permitido desenvolver este projeto e por ter acompanhado o seu decorrer.

Aos membros do Grupo de Oncologia Molecular e Patologia Viral do Centro de Investigação do IPO-Porto, em especial à Mafalda e à Sara por toda a companhia até às horas que fossem preciso e por me acompanhem nos devaneios mentais ocasionais. Agradeço pela grande ajuda na parte laboratorial (de certeza que se ficarem míopes não é por minha causa) e pelas gargalhadas ao som de Quim Barreiros.

Ao Marco, que nunca se importou de ficar a olhar para mim enquanto eu escrevia apesar de estar um belo dia lá fora. Obrigada pela paciência de santo.

Aos meus amigos de sempre que estão sempre prontos para um jantar para desanuviar só porque sim. À Clara, à Cristina e à Rita pelas sessões de filmes em que eu acabava a dormir passados cinco minutos, pela organização das viagens mais inesperadas em tempo recorde e pelas sessões de Skype cada vez mais internacionais.

Aos meus pais por me deixarem fazer aquilo que mais gosto e à minha irmã por ouvir as minhas dúvidas existenciais. Por fim, também tenho de agradecer ao meu cão por estar sempre pronto para me aquecer as pernas durante das tardes de escrita ao fim de semana.

Apoio financeiro e divulgação dos resultados

Este projeto foi financiado pela Sociedade Portuguesa de Gastrenterologia (SPG), sendo que os estudos descritos nesta tese resultam da colaboração entre o Grupo de Oncologia Molecular e Patologia Viral do Centro de Investigação, o Serviço de Gastrenterologia e o Serviço de Anatomia Patológica do Instituto Português de Oncologia do Porto (IPO-Porto).

Parte dos resultados aqui descritos foram já apresentados sob a forma de póster no YES (*Young European Scientist*) Meeting 2019, Porto (ver *Appendix*). Mais ainda, as publicações que irão integrar os resultados que derivam deste projeto de Mestrado encontram-se em fase de preparação.

Resumo

O cancro gástrico (CG) é o quinto cancro mais comum globalmente e a terceira principal causa de morte relacionada a esta doença. A taxa de incidência de CG em Portugal, especialmente na zona Norte do país, é uma das mais elevadas da Europa. A Prostaglandina E_2 (PGE_2) desempenha um papel importante nas vias carcinogénicas mais relevantes e os seus níveis são regulados principalmente pela cicloxigenase-2 (COX-2) e pela *multidrug resistance protein 4* (MRP4), responsáveis pela síntese da PGE_2 e pelo seu transporte para o meio extracelular, respetivamente, e pela 15-hidroxiprostaglandina desidrogenase (15-PGDH) e pela proteína transportadora de prostaglandinas (PGT), responsáveis pela sua inativação. Apesar de existirem características genéticas e moleculares distintas entre populações étnicas, a maioria dos estudos publicados foca-se em populações Asiáticas. Devido ao facto de um programa de rastreio em massa, como o que é observado no Japão, ser injustificado em países com taxas de incidência mais baixas, a identificação de fatores de risco que possam ser utilizados como biomarcadores para um rastreio personalizado e vigilância poderá ser a melhor opção.

Assim, o nosso objetivo principal foi a caracterização do perfil genómico dos quatro genes que codificam as proteínas mencionadas anteriormente (*PTGS2, ABCC4, HPGD,* e *SLCO2A1*, respetivamente) associado a risco para CG utilizando uma abordagem de tagSNPs numa população Caucasiana da região Norte de Portugal. Adicionalmente, explorámos a expressão de mRNA desses genes em CG e avaliámos a influência dos polimorfismos mais relevantes na sua expressão. Neste estudo preliminar, 48 tagSNPs foram genotipados em 222 amostras fixadas em formaldeído e embebidas em parafina (FFPE) de pacientes com confirmação histológica de CG do tipo intestinal e em 476 controlos livres de cancro utilizando a tecnologia *MassARRAY® iPLEX Gold* ou PCR em tempo real, através de discriminação alélica. Foi possível extrair RNA de 94 amostras de mucosa aparentemente "normal" e de 89 amostras de mucosa tumoral e a sua expressão foi avaliada por PCR em tempo real.

Oito tagSNPs foram identificados como biomarcadores de suscetibilidade para o desenvolvimento de CG: rs689466 no gene *PTGS2*; rs1678374, rs1678405, e rs1751031 no gene *ABCC4*; rs2303520 no gene *HPGD*; e rs10935090, rs11915399, e rs9821091 no gene *SLCO2A1*. Para além disso, os polimorfismos rs2303520 e rs11915399 demonstraram ter influência na expressão dos genes *HPGD* e *SLCO2A1*, respetivamente, suportando os dados epidemiológicos. A análise *multifactor dimensionality reduction* (MDR) identificou o melhor modelo interativo de três fatores, constituído por idade,

rs689466 e rs178374. Este modelo apresentou a precisão de validação cruzada mais elevada (80,7%) e uma consistência de validação cruzada de 8/10 ($P<0,0001$) e estava associado a um aumento no risco para CG de 17,6 vezes. Encontrámos níveis aumentados de expressão de mRNA da COX-2 e uma subexpressão dos genes que codificam as proteínas 15-PGDH e PGT em mucosa tumoral comparado com a mucosa normal, numa maneira independente do género. Nas mulheres, observámos uma subexpressão do gene *ABCC4* em mucosa tumoral quando comparado com os homens (1,13±0,17 vs 1,85±1,17; $P=0,038$).

Concluindo, os nossos resultados enfatizam a importância da via da PGE_2 na carcinogénese gástrica e o seu papel na modulação da suscetibilidade para CG nas populações Caucasianas. Para além disso, se suportado por estudos futuros, a identificação de biomarcadores genéticos poderá permitir a identificação de indivíduos com risco aumentado que podem ser direcionados para rastreio personalizado e/ou uma abordagem quimiopreventiva.

Palavras-chave

Cancro gástrico, expressão de mRNA, polimorfismos genéticos, tagSNPs, cicloxigenase-2, *multidrug resistance protein 4*, 15-hidroxiprostaglandina desidrogenase, proteína transportadora de prostaglandinas, *PTGS2, ABCC4, HPGD, SLCO2A1*

Summary

Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related deaths. GC incidence rate in Portugal, especially in the Northern region of the country, is one of the highest in Europe. Prostaglandin E_2 (PGE_2) plays a key role in virtually all hallmarks of cancer and its levels are mainly regulated by cyclooxygenase-2 (COX-2) and multidrug resistance protein 4 (MRP4), responsible for PGE_2 synthesis and transport to the extracellular milieu, respectively, and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and prostaglandin transporter (PGT), responsible for its inactivation. Even though there are distinct genetic and molecular signatures across ethnic populations, most published studies are focused on Asian populations. Due to the fact that a mass screening program, such as the one observed in Japan, is unwarranted in countries with lower incidence rates, the identification of risk factors that can be used as biomarkers for personalized screening and surveillance might be the best option.

Thus, our main objective was to characterize the genomic profile of the four genes that encode the proteins mentioned above (*PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1*, respectively) associated with GC risk using a tagSNP approach in a Caucasian population from the Northern region of Portugal. Additionally, we further explored the mRNA expression of those genes in GC and assessed the influence of the most relevant polymorphisms in their expression. In this preliminary study, 48 tagSNPs were genotyped in 222 formalin-fixed paraffin-embedded (FFPE) samples from patients with histological confirmation of intestinal-type GC and 476 cancer-free controls using the MassARRAY® iPLEX Gold Technology or by Real-Time PCR through allelic discrimination. The RNA was successfully extracted from 94 “normal”-appearing mucosa samples and 89 tumor samples and its expression was assessed by Real-Time PCR.

Eight tagSNPs were identified as susceptibility biomarkers for GC development: rs689466 in *PTGS2* gene, rs1678374, rs1678405, and rs1751031 in *ABCC4* gene, rs2303522 in *HPGD* gene, and rs10935090, rs11915399, and rs9821091 in *SLCO2A1* gene. Furthermore, rs2303520 and rs11915399 polymorphisms were found to have an influence on the expression of *HPGD* and *SLCO2A1* genes, respectively, supporting the epidemiological data. The multifactor dimensionality reduction (MDR) analysis identified an overall three-factor best interactive model composed by age, rs689466, and rs1678374. This model presented the highest cross-validation (CV) accuracy (80.7%) and a CV consistency (CVC) of 8/10 ($P<0.0001$) and was associated with a 17.6-fold increase in GC risk. We found increased levels of COX-2 mRNA expression and a downregulation of the

15-PGDH and PGT encoding genes in tumorous mucosa compared to the normal mucosa in a gender-independent manner. In females, we observed an *ABCC4* downregulation compared to males in tumoral mucosa (1.13±0.17 vs 1.85±1.17; P=0.038)

In conclusion, our results highlight the importance of the PGE_2 pathway in gastric carcinogenesis and its role in the modulation of GC susceptibility in Caucasian populations. Furthermore, if supported by further studies, the identification of genetic biomarkers might allow the identification of increased risk individuals that can be targeted for personalized screening and/or chemopreventive approach.

Keywords

Gastric cancer, genetic polymorphisms, genetic susceptibility, tagSNPs, mRNA expression, cyclooxygenase-2, multidrug-resistance protein 4, 15-hydroxyprostaglandin dehydrogenase, prostaglandin transporter, *PTGS2, ABCC4, HPGD, SLCO2A1*

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List of abbreviations

A	Adenine
AA	Arachidonic Acid
ABC	ATP-Binding Cassette
ABCC4	ATP-Binding Cassette Sub-Family C Member 4
AJCC	American Joint Committee on Cancer
C	Cytosine
CDH1	E-cadherin
CI	Confidence Interval
CIN	Chromosomal Instability
COX	Cyclooxygenase
CV	Cross-Validation
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EP	Prostaglandin E_2 Receptor
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
G	Guanine
GC	Gastric Cancer
GEJ	Gastroesophageal Junction
GS	Genomic Stability
GWAS	Genome-Wide Association Studies
H. pylori	*Helicobacter pylori*
HWE	Hardy-Weinberg Equilibrium
IARC	International Agency for Research on Cancer
IL	Interleukin
IPO-Porto	*Instituto Português de Oncologia do Porto*
LD	Linkage Disequilibrium
MDR	Multifactor Dimensionality Reduction
MRP4	Multidrug Resistance Protein 4
MSI	Microsatellite Instability

NSAID	Non-Steroidal Anti-Inflammatory Drug
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGT	Prostaglandin Transporter
PTGS2	Prostaglandin Endoperoxide H Synthase 2
RNA	Ribonucleic Acid
RT	Reverse Transcription
SD	Standard Deviation
SLCO2A1	Solute Carrier Organic Anion Transporter Family, Member 2A1
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
T	Thymine
TCGA	The Cancer Genome Atlas
TMD	Transmembrane Domain
TNF	Tumor Necrosis Factor
TXA	Thromboxane
15-PGDH	15-Hydroxyprostaglandin Dehydrogenase

1. INTRODUCTION

1.1. Epidemiology of gastric cancer

1.1.1. Incidence, distribution, and survival

Cancer is still considered a life-threatening disease nowadays, despite all the major advances in diagnosis and treatment, being one of the main causes of death before the age of 70 around the world [1]. According to the International Agency for Research on Cancer (IARC), gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related deaths, being responsible for more than 1 million new cases and 780 000 deaths in 2018 [1]. There is a geographically heterogenous distribution of GC around the globe, with the highest incidence and mortality rates occurring in Asia (over 70% in both cases), followed by Europe, Latin America and Caribbean [1]. Lower rates are found in North America, Africa, and Oceania [1]. The absolute and relative numbers of individuals diagnosed and GC-associated deaths in 2018 are summarized in **Table 1** [1, 2].

Table 1. Gastric cancer absolute incidence and mortality in 2018 stratified by population, including both sexes and all ages. Source: Globocan 2018

Population	Incidence		Mortality	
	Number	Percentage (%)	Number	Percentage (%)
Asia	769 728	74.5	584 375	74.7
Europe	133 133	12.9	102 167	13.1
Portugal	**2 885**	**(2.2*)**	**2 275**	**(2.2*)**
Latin America and the Caribbean	67 058	6.5	51 914	6.6
Africa	31 148	3	28 707	3.7
North America	29 275	2.8	13 403	1.7
Oceania	3 359	0.32	2 119	0.27
Total	**1 033 701**	**100**	**782 685**	**100**

*Percentage representing absolute incidence and mortality values in Portugal in relation to Europe.

The annual reports on cancer incidence and mortality in Northern Portugal, including Braga, Bragança, Porto, Viana do Castelo, and Vila Real, are published by the North Region Cancer Registry of Portugal (RORENO), which is a population-based cancer registry that covers over 3 million Portuguese people [3]. In this region of the country (**Figure 1**), GC remains the fourth most common cancer and, even though its incidence rates have been declining, they remain one of the highest in Europe [3, 4].

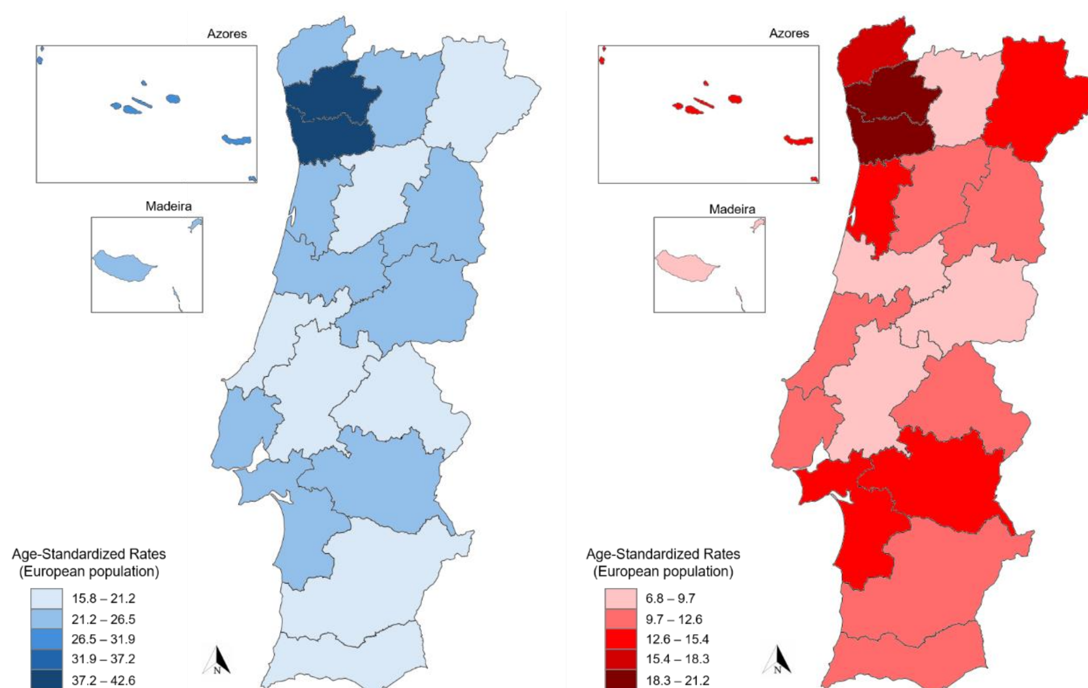

Figure 1. Geographical distribution of GC in Portugal in males (left) and females (right). Data from *Registo Oncológico Nacional 2010.*

GC survival is higher in Portugal when compared to the European mean, probably because of the high frequency of tumors diagnosed with a better prognosis [5]. Nevertheless, it is difficult to control the progression of the disease through early diagnosis due to its asymptomatic nature and the lack of specificity of the symptoms, resulting in around 70% of the patients reaching a more advanced-stage disease [6-8]. The highest 5-year survival rates are found in Asia, with highlight to Republic of Korea and Japan (over 70%) [1]. This high rate may be due to the efficiency of the screening programs for GC in Asia, which allows a higher number of diagnosed cases in early stages of cancer [9]. Patients diagnosed in advanced stages of the disease who receive conventional therapies, such as surgery, radiotherapy, and/or chemotherapy, have a poor prognosis, with most Western countries having 5-year survival rates lower than 30% [10]. On the opposite side, patients with an early-stage diagnosis have a better prognosis and the survival rate exceeds 90% [11].

In Asian countries like Japan, that have a high incidence of this disease, the detection of GC in early stages is provided by mass screening of the population [9]. Still, such strategy is costly and unwarranted for countries with a lower incidence rate, like the United States of America or most European countries [9]. In Portugal, a country with an intermediate GC risk, Areia *et al.* [12, 13] reported that endoscopic screening is cost-

effective only if combined with a scheduled colonoscopy after a positive fecal occult blood test.

Despite the declining age-standardized incidence, the number of newly diagnosed cases of gastric cancer is still increasing, probably due to the aging of the population [14]. This disease is expected to be responsible for over 1 300 000 deaths worldwide in 2040 [1].

1.1.2. Known risk factors

Gastric carcinogenesis is a multifactorial process, involving numerous dietary and nondietary factors, as well as genetic components (**Figure 2**), demonstrated by the fact that GC development occurs only in a small proportion of people exposed to the environmental risks [15]. GC can be associated with specific mutational profiles (hereditary) or, most frequently (over 90% of cases) and what will be the focus of this thesis, sporadic, deriving from the accumulation of changes in phenotype and genotype [16]. These changes may be triggered by other conditions, like gastritis, which can be a result of infection by *H. pylori* [16].

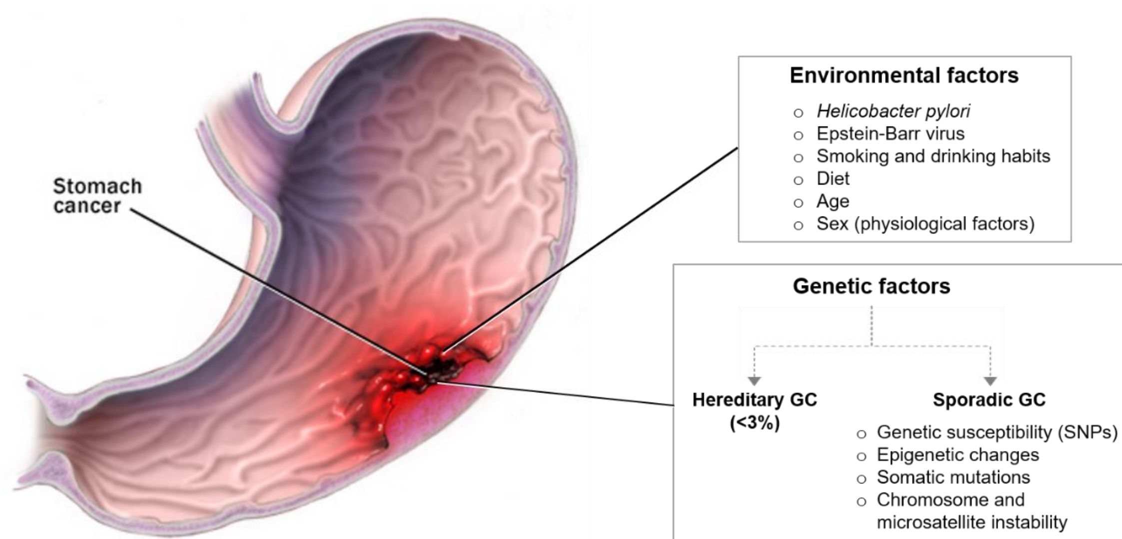

Figure 2. **Known risk factors for GC onset and development.** Adapted from [17].

***Helicobacter pylori* (*H. pylori*)** is a Gram-negative spiral bacterium with the ability to selectively colonize the human gastric mucosa and affect the immune response developed by the host [16]. The infection by this microorganism is usually acquired during childhood and might persist for many years, even decades, unless *H. pylori* is eradicated [16]. Virtually everyone infected with this bacterium develops gastritis and the long-term interaction with the host has biological costs, such as chronic inflammation, known to be

a risk factor for the onset of more serious conditions, gastric adenocarcinoma, and non-Hodgkins lymphoma of the stomach [18]. Thus, *H. pylori* most likely plays an important role in early stages of gastric carcinogenesis, being necessary, but not sufficient, for the development of GC [18]. There are different strains of this pathogen with distinct virulence, with some being more likely to cause GC, namely the strains positive for the virulence factor cytotoxin-associated gene A (cagA) [9].

H. pylori infection was recognized by the IARC as a type I carcinogen in 1994 and it is associated with both intestinal and diffuse types, causing 65-80% of all GC cases [9, 16]. This number may be underestimated due to the insufficient sensitivity of the methods used to access the infection, like the enzyme-linked immunosorbent assay (ELISA) [9]. *H. pylori* increases malignancy risk up to six-fold and is considered a major risk factor for non-cardia GC, being responsible for 75% of the cases, but not for cardia GC, at least in Western countries [9, 19]. The declining incidence of non-cardia GC may be related to better sanitation and the use of antibiotics, which are responsible for decreasing *H. pylori* prevalence [9]. The anatomic classification of gastric tumors is described below.

Even though *H. pylori* infection is so common, only a small percentage of infected people develop more serious health issues, suggesting that disease progression involves specific and dynamic interactions between the pathogen and the host [18]. Moreover, it is intensified by other risk factors, such as the dietary factors mentioned below [18]. Limitation of the bactericidal effects of pro-inflammatory molecules, suppression of immune responses, and variation of the antigens present at the cell surface are some strategies evolved by the bacterium to allow its persistence in the host [18]. Additionally, *H. pylori* may act through various mechanisms, including induction of hyperproliferation, increase in the production of reactive oxygen species (ROS) and nitric oxide (NO), and interference with antioxidant functions [15].

An association between **Epstein-Barr virus (EBV)** and a rare subtype of GC, the lymphoepithelioma, was first reported by Burke *et al.* [20], but further studies found links between EBV and other more common subtypes [21]. This virus is one of the most common human herpesviruses, infecting over 90% of the population by adulthood and can be found in malignant epithelial cells of gastric carcinomas [22].

Dietary factors include high consumption of salt-rich food, with salt causing direct damage to the gastric mucosa that can lead to gastritis or other gastric problems, and the consumption of food subjected to cooking processes such as pickling or grilling, due to the formation of N-nitroso compounds [8, 9]. Moreover, studies have suggested that diets rich in meat, which are common in Europe, correlate with distal GC and this association is even stronger in people infected with *H. pylori* [16]. The consumption of fruits and vegetables, rich in vitamin C, carotenoids, folate, and phytochemicals, responsible for the regulation

of xenobiotic-metabolizing enzymes and for the inhibition of carcinogenesis, milk, green tea, aspirin, and statins have been shown to have a protective role in GC [8, 9, 16]. Overall, adherence to a Mediterranean diet and diets rich in antioxidants and fiber content are associated with lower GC risk, whereas diets with a more Western-pattern may increase that risk [9].

Smoking and drinking habits are also associated with higher GC risk [16]. Tobacco may be responsible for the onset of gastric diseases like gastritis, ulcers and both proximal and distal GC and has even been classified as the most relevant behavioral risk factor for GC by Ladeiras-Lopes *et al.* [16, 23]. Both intensity and duration of smoking habits in European males and females have been associated with proximal GC in particular [24]. Moreover, smoking cessation prior to GC surgery, especially for heavy smokers, has been suggested to reduce post-operative complications [25]. Regarding alcohol, a strong association was found between cardia cancer and heavy drinking. However, light to moderate consumption led to an insignificant increase in the risk [16, 26].

The observed decline in stomach cancer incidence and mortality in the last decades is attributed to the increase of fruit and vegetable consumption, and the decrease in smoking, salt intake, and *H. pylori* infection [4]. However, *H. pylori* prevalence appears to be persistently high in Northern Portugal, so the former factors may play a bigger role in the declining GC rates [3, 4]. Moreover, a study by Soerjomataram *et al.* [27] reports that, if countries from South and Eastern Europe with high GC rates, like Portugal, Lithuania, and Estonia, presented the same smoking and *H. pylori* patterns as Northern countries, such as Denmark and Sweden, a big percentage of the cases observed would be avoidable [28]. Moreover, besides endoscopic surveillance, *H. pylori* eradication is the most important strategy to prevent GC, particularly in first-degree relatives of patients, possibly preventing the progression to intestinal metaplasia [14]. This measure could result in a reduction by 30% of the risk of developing GC and, additionally, decrease the risk of tumor recurrence [29].

In regard to **sex**, the age-standardized incidence rate is twice as high in men (15.7%) as in women (7.0%) [1]. The reason for this difference is not clear, however, some environmental or occupational exposures may play a role, as well as physiological differences [9]. Hormones like estrogens may play a protective role against GC during the fertile years of the women [9]. Therefore, delayed menopause and increased fertility may lower the risk of GC development [9].

GC is considered an “environmental cancer” and, as such, the risk of gastric carcinoma is lower in young people, increasing gradually with age [16]. However, recent studies reveal that the incidence of GC is increasing in people of young age [6]. Moreover, it has been noted that there is a significant difference between ethnic groups within the

same region, with GC affecting more Hispanics, African Americans and Native Indians than Caucasians, for example [16]. This might be due to the overlapping disparities regarding socioeconomic status and, consequently, *H. pylori* prevalence, an infection known to be an important risk factor for GC, as previously mentioned [9, 15, 16]. Comparing racial groups, cardia GC is twice as common among Caucasians, whereas non-cardia GC is half as common [9].

Other risk factors include **low socioeconomic status** and, consequently, low education and low income, **radiation**, which has been related to GC in a dose-response manner [9], and **history of stomach disorders** [8]. Some factors, like **obesity** and gastroesophageal reflux disease (**GERD**), are associated with a local-specific subtype of cancer, cardia GC, but not non-cardia GC [9].

The **genetic component** is also an important risk factor to take into consideration. People with a family history of GC are two to ten times more likely to develop cancer in the stomach [16]. However, most familial cases are considered sporadic and seem to be influenced by the environmental factors mentioned above [16]. Some common genetic variants, namely single nucleotide polymorphisms (SNPs), have been suggested as genetic susceptibility biomarkers of GC development [2]. They are responsible for the regulation of multiple pathways that occur during gastric carcinogenesis, modulating the effects of exposure to the environmental risk factors [30]. There has been increasing interest in these gene-environment interactions that might explain the heterogenic distribution of GC incidence around the world [15].

Genetic polymorphisms, particularly in genes related to inflammation, have been implicated in the initiation and progression of the tumor and identified as potential risk biomarkers for the development of GC, as highlighted in the field synopsis followed by meta-analysis published by Mocellin *et al.* in 2015 [2, 31]. For example, both interleukin (IL)-1β and tumor necrosis factor (TNF)-α are pro-inflammatory cytokines involved in the suppression of gastric acid production and polymorphisms in their genes are associated with a higher risk due to their pro-inflammatory nature [16]. On the other hand, the anti-inflammatory cytokine IL-10 counteracts the previous effect by influencing acid production [16]. A number of studies have suggested that some *IL10* gene polymorphisms may be associated with GC among Asians, but not Caucasians, and that might indicate the involvement of different mechanisms behind the regulation of this gene [2].

Most genetic susceptibility studies in GC have been performed in Asian populations, namely Japanese, Korean, and Chinese, including genome-wide association studies (GWAS), a reflection of the high incidence and mortality rates observed in those countries [9]. The study by Mocellin *et al.* [31] reports the predominant role that chromosome 1 might play in the susceptibility of GC, defending that this region may

represent a promising target for further research in the GC field. Moreover, the authors validated the hypothesis that there are distinct molecular pathways regulating the GC genetic susceptibility across different ethnicities [31]. In fact, the highest rates found in Asia can be attributed, at least in part, to the prevalence of *H. pylori* infection and to differences in the distribution of genetic polymorphisms in distinct populations [31]. Kamangar *et al.* [32] explored the association between polymorphisms in inflammation-related genes and the risk of developing GC in a Finnish population. Even though other published studies reported the association between pro-inflammatory genetic polymorphisms and a greater GC risk, these observations were not replicated among Finnish male smokers and the authors conclude that these findings may implicate different etiologies in different populations [32]. On the other hand, a series of meta-analyses performed by Persson *et al.* [33] point to a clear increased risk of GC associated with *IL1RN2* genetic variants. This association was observed for both intestinal and diffuse cancers, as well as distal and proximal cancer, the latter to a lesser extent, but it appeared to be confined to non-Asian populations [33]. Regarding Asian populations, the authors found the strongest association with the *IL1B-31* gene [33]. Overall, the authors argue the importance of data stratifications by parameters like GC subtype, site, geographic location and *H. pylori* infection [33].

1.2. Natural history of gastric cancer

1.2.1. Stomach anatomy

The human stomach can be divided into distinct anatomic regions based on surgical markers (**Figure 3**): the cardia, the fundus, the body or corpus, and the antrum [35]. **Cardia** is the most proximal portion of the stomach, following the gastroesophageal junction (GEJ) [34]. Extending above that junction is the **fundus**, forming a sharp angle with the distal esophagus (cardiac notch) [35]. The **body or corpus** is located between the fundus and the lower region of the stomach, forming the lesser and greater curvature and being marked distally by the **angularis incisura**, a notch on the lesser curvature near the pyloric end [34, 35]. The last segment of the stomach is the **antrum**, which is not anatomically distinguishable but extends from the corpus to the

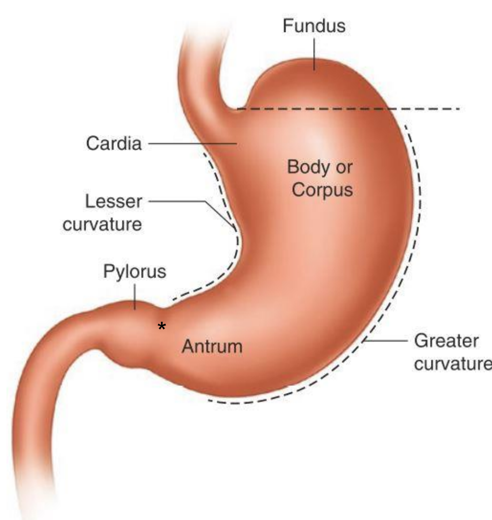

Figure 3. Topography of the human stomach. The region marked with a * corresponds to incisura angularis. Adapted from [34].

pyloric canal (**pylorus**) [35]. Separating the stomach from the duodenum is the pyloric sphincter, a thick valve of smooth muscle [34, 35].

Externally, the angularis incisura separates the acid-secreting regions, fundus, and corpus, from the non-acid secreting antrum, which is composed of an alkaline-secreting epithelium and gastrin-secreting G-cells [36].

1.2.2. Gastric cancer classification and carcinogenesis cascade

Gastric carcinogenesis is a multistep and histologically heterogenous process, encompassing a group of malignant lesions at the epithelial level [37]. Over 90% of all primary gastric malignancies are represented by **gastric adenocarcinoma**, while the others correspond to stromal tumors (GIST) and other types of GC [7].

The World Health Organization (WHO) recognizes five main types of gastric adenocarcinomas according to their morphological patterns: papillary, tubular, mucinous, poorly cohesive (including signet-ring cell type), or mixed adenocarcinoma [38].

Histologically, the classification more frequently used is the one proposed in 1965 by **Laurén** [39], which distinguishes diffuse from intestinal-type gastric adenocarcinomas. The former accounts for 30% of cases and, overall, is characterized by the lack of intercellular adhesion, namely because of an inactivating mutation in the gene encoding the glycoprotein E-cadherin, also known as cadherin-1 (*CDH1*) [40]. In this thesis, we will focus on intestinal-type GC, which accounts for 50-70% of all cases and represents the most diagnosed histological subtype of GC, with its geographical distribution overlapping with that of *H. pylori* prevalence [16]. This histological type of GC is more prevalent among sporadic cancers and its carcinogenesis, first proposed by **Correa** [41] and represented in **Figure 4**, is progressively associated with corpus-dominated gastritis, intestinal metaplasia, dysplasia, and adenocarcinoma [8, 29]. Atrophic gastritis is defined by most pathologists as the loss of specialized glandular tissue, whereas intestinal metaplasia corresponds to the replacement of the original gastric glands by the absorptive and goblet cells that characterize the intestine, accompanied by inflammation in the lamina propria [29]. Gastric atrophy is associated with GC much more consistently and, for that reason, comparing to intestinal metaplasia, appears to be a better indicator of GC risk [29]. Regarding dysplasia, it is a premalignant lesion that can be classified as low-grade or high-grade dysplasia and the latter bears a higher risk for GC development [42].

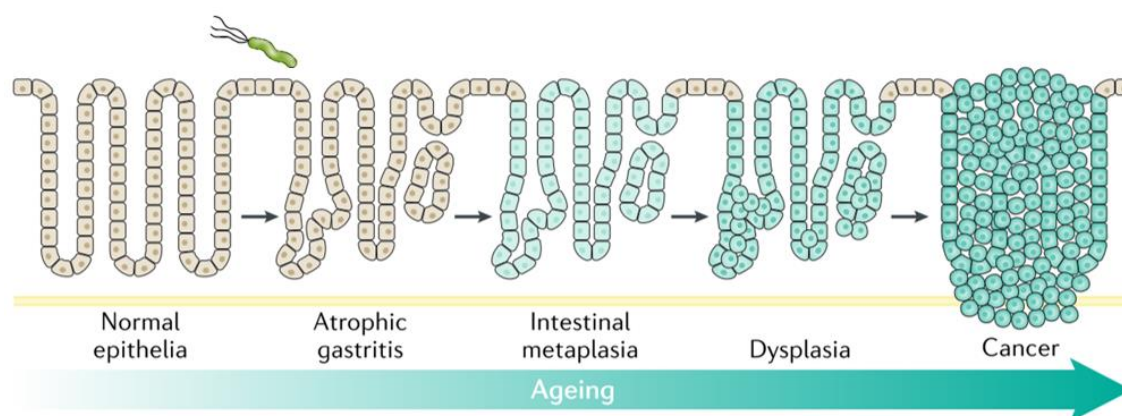

Figure 4. Progression of intestinal-type gastric adenocarcinoma. Infection by *H. pylori* usually occurs in an early stage of life and over a period of weeks, leading to gastritis. Adapted from [43].

Anatomically, gastric tumors are typically classified as proximal (or cardia), and distal (or non-cardia), the latter commonly associated with *H. pylori* infection [29]. GC cases can also be classified as early or advanced according to their stage [6]. While the early GC cases are limited to the mucosa or submucosa, regardless of lymph node invasion, the more advanced stages infiltrate into or beyond the subserosa layer and are able to metastasize [6, 7]. Furthermore, cancer able to extend beyond the submucosa and invade the muscular layer of the stomach is classified as middle GC [6].

1.2.3. Gastric cancer staging

Cancer staging is a crucial tool for defining prognosis, understanding the probability of overcoming the disease, and determining the best approach for treatment [44]. The TNM classification is used worldwide and classifies the tumor based on its extent (T), the invasion of regional lymph nodes (N), and the existence of distant metastases (M) [44]. The most recent edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual distinguishes three-stage grouping systems: pathological staging (pTNM), dependent on clinically acquired data, clinical staging (cTNM), based on evidence present before therapy, and post neoadjuvant therapy staging (ypTNM), which reports the response of the tumor to a preoperative therapy [44]. The AJCC TNM definitions of primary tumor (T) are based on the depth of tumor invasion, ranging between T0 (no evidence of primary tumor) and T4 (invasion of the serosa or adjacent structures), with both T1 and T4 categories being subdivided into a and b; N reports the number of positive of lymph nodes and includes the following criteria: N0 (no lymph node metastasis), N1 (one or two metastasized nodes), N2 (three to six metastasized nodes), and N3 (metastasis in seven or more lymph nodes), which can subdivided into N3a (between seven and fifteen) and

N3b (sixteen or more); distant metastasis (M) is defined by either one of two categories: M0 (no distant metastasis) or M1 (distant metastasis) [44]. The TNM stage grouping is summarized in **Table 2.**

Table 2. AJCC pathological stage groups. In stage 0, Tis corresponds to carcinoma *in situ*. Source: AJCC Cancer Staging Manual [44].

Stage	T	N	M	Stage	T	N	M
0	Tis	N0	M0	**IIIA**	T2	N3a	M0
IA	T1	N0	M0		T3	N2	M0
IB	T1	N1	M0		T4a	N1, N2	M0
	T2	N0	M0		T4b	N0	M0
IIA	T1	N2	M0	**IIIB**	T1, T2	N3b	M0
	T2	N1	M0		T3, T4a	N3a	M0
	T3	N0	M0		T4b	N1, N2	M0
IIB	T1	N3a	M0	**IIIC**	T3, T4a	N3b	M0
	T2	N2	M0		T4b	N3a, N3b	M0
	T3	N1	M0	**IV**	Any T	Any N	M1
	T4a	N0	M0				

1.3. Molecular characterization of gastric adenocarcinoma

In order to identify dysregulated pathways and potential drivers of this type of cancer, The Cancer Genome Atlas (TCGA) described a molecular classification which defines four major subtypes of GC, as illustrated in **Figure 5**: chromosomal instability (CIN), EBV-infected (EBV), microsatellite instability (MSI), and genomic stability (GS) [45]. Most tumors evaluated in this report exhibited CIN (50%), followed by MSI (22%), GS (20%), which was found to be enriched in diffuse-type GC, and EBV-positivity (9%) [45]. Regarding localization, every subtype was found throughout the stomach, but CIN tumors predominated in the cardia and gastroesophageal junction, whereas EBV tumors were mainly found in the fundus or corpus [45].

EBV tumors are characterized by a mutation in the *PIK3CA* gene and, consequently, PI(3)-kinase inhibition, and a higher prevalence of deoxyribonucleic acid (DNA) hypermethylation, resulting in *CDKN2A* silencing [45]. On the other hand, besides hypermethylation, namely in the *MLH1* gene, MSI tumors showed high mutation rates [45]. A high percentage of CIN tumors (71%) presented *TP53* mutations and 37% of the genomically stable (GS) tumors showed *CDH1* germline mutations, associated with a

subtype of diffuse GC [45]. Mutations in *RHOA* gene were found almost exclusively in the latter molecular subtype [45].

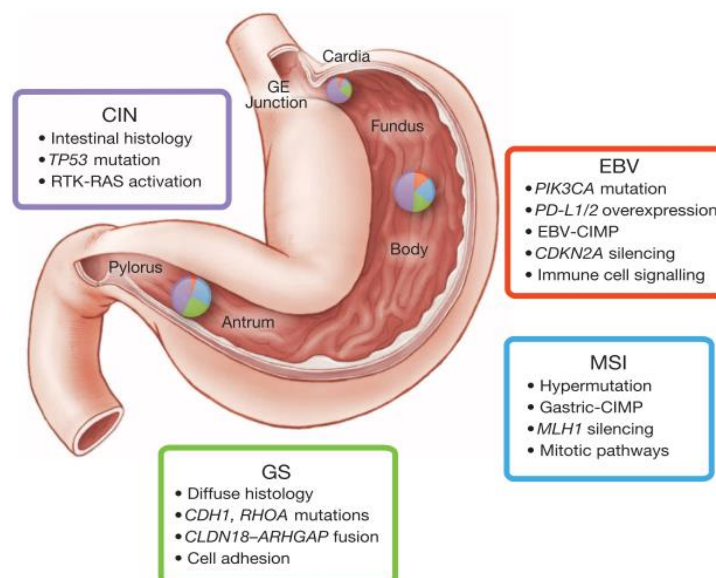

Figure 5. The major molecular subtypes of GC according to TCGA and their key features. From [45].

1.4. The COX-2/PGE_2 pathway

Prostaglandins (PGs), including PGD, PGE, and PGF, are part of a big family of biologically active lipids named prostanoids and play important roles in the development of many types of cancer, including GC [46, 47]. These lipids containing 20 carbons can be synthesized intracellularly from a variety of precursors, being arachidonic acid (AA), the precursor of series 2 prostanoids, the most predominant and important in humans [46]. Other members of the prostanoid family include prostacyclin (PGI) and thromboxane (TXA) which are also involved in physiological processes such as gastric cytoprotection, inflammation, and modulation of vascular tone [48].

Under basal conditions, AA is virtually undetectable, but it can be mobilized from the plasma membrane in response to several stimuli, namely growth factors, hormones, and cytokines [48]. Eicosanoids are active metabolites generated by the AA pathway and have been shown to be involved in several pathologies, namely inflammation and cancer [48]. These metabolites result from the action of different groups of enzymes: epoxygenases (cytochrome P450), lipoxygenases (LOX), and cyclooxygenases (COX) [48].

1.4.1. Cyclooxygenase-2 (COX-2)

Cyclooxygenase 1 or 2 (COX-1 or COX-2), also known as PG endoperoxide H synthase 1 or 2 (PTGS1 or *PTGS2*), is an approximately 72 kDa protein that catalyzes the rate-limiting step in PG synthesis, the addition of molecular oxygen into AA [48, 49]. The resulting PGG_2, an unstable intermediate, is subsequently and rapidly converted to PGH_2 by the peroxidase activity of COX-2 [46]. Thus, this enzyme has a dual enzymatic activity: the cyclooxygenase activity is responsible for giving PG its characteristic hairpin structure by creating a ring at the midpoint of the AA molecule, whereas the peroxidase activity forms PGH_2 by reducing a hydroperoxide group to a hydroxyl [50].

The two isoforms of COX are coded by different genes but share sequence homology and catalytic activities [50]. COX-1, encoded by the *PTGS1* gene, is often considered the "housekeeping" isoform, as it is constitutively expressed in most tissues and modulates important functions for homeostasis and the maintenance of basal PG levels [50, 51]. On the other hand, COX-2, encoded by *PTGS2* gene, is usually undetectable in most normal tissues, with a few exceptions like the kidney, central nervous system, and seminal vesicles [51]. Its levels can rapidly increase in response to specific stimuli, such as hormones, hypoxia, growth factors, and cytokines, namely TNFα and IL-1β, thus being referred to as the "inducible" form of COX [48, 50, 52]. These two proteins, like other enzymes involved in PG synthesis, are associated with the nuclear envelope and endoplasmic reticulum membranes, being located in the perinuclear area [50].

Upregulation of COX-2 has been observed in cancerous and precancerous tissues, resulting in the accumulation of PGs, particularly PGE_2, in the extracellular milieu, and is associated with poor prognosis [53, 54]. Moreover, some cancer-causing agents, like tobacco smoke, UV irradiation, and microbial agents, have been shown to induce COX-2 expression [55]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) that target primarily the COX enzymes, such as aspirin, has been reported in cancer prevention [56, 57]. In spite of being effective, these agents are associated with side effects, as they compromise the gastrointestinal tract [56]. Therefore, new enzymatic targets within this pathway have been studied and developed, including the PG terminal synthases [57].

The rate-limiting product of this pathway, PGH_2, is modified by specific enzymes with synthase activity via reduction, isomerization, or rearrangement, generating PGs and TXA with different biological properties [48]. The best-known and most abundant PG in the human body is PGE_2, playing important roles in several systems, including cardiovascular, reproductive, renal, and gastrointestinal [46]. Additionally, it is synthesized by monocytes/macrophages, mast cells, and platelets and is the main mediator of inflammation [46, 48]. The pleiotropic effects of PGE_2 appear to affect virtually all the

hallmarks of cancer (evasion of apoptosis, insensitivity to anti-growth signals, self-sufficiency, sustained angiogenesis, limitless replicative potential, tissue invasion, and metastasis) and, as such, it has been shown to be involved in the development of human malignancies [51, 58]. Moreover, PGE_2 also has strong immunosuppressive effects, promoting tumor growth by allowing it to evade immune surveillance [48]. In a tumor, the activation of the enzymes responsible for PGE_2 production leads to an increased concentration of this PG in tumor cells, endothelial cells, fibroblasts, and immune cells [59]. A representation of the major players in PGE_2 levels regulation is displayed in **Figure 6**.

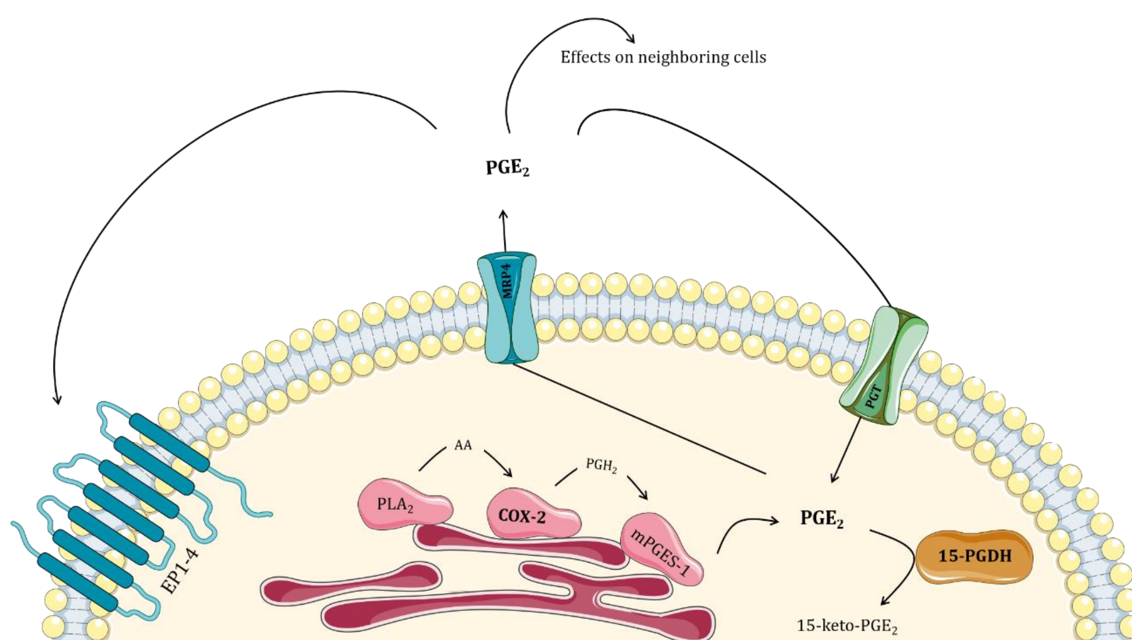

Figure 6. The PGE_2 pathway. PGE_2 precursor, AA, is released from the membrane by phospholipase A_2 (PLA_2) and then converted to PGH_2 by COX-2. This intermediate is further converted to PGE_2 by a terminal synthase, mPGES-1. That PG can then be transported out of the cell by MRP4, where it is able to interact with specific prostanoid receptors (EP1-EP4) and exert its effects in an autocrine or paracrine manner. On the other hand, PGE_2 can return to the intracellular environment via PGT and be inactivated by 15-PGDH, originating 15-keto-PGE_2.

1.4.2. Multidrug resistance protein 4 (MRP4)

After synthesis, in its active form, PGE_2 can either cross the cell membrane by simple diffusion in response to a concentration and electrochemical gradient or be transported by members of the ATP-binding cassette (ABC) transporter superfamily [50]. However, simple diffusion occurs poorly due to the negatively charged nature of these bioactive molecules at physiological pH [46]. The ABC transporter family is subdivided into seven different subfamilies, from A to G, based on sequence homology [60]. The C subset, also known as the multidrug-resistant protein (MRP) subfamily, has attracted more

attention due to its members location in the plasma membrane and ability to pump out many structurally different molecules, either endogenous or exogenous [60]. They are known to be involved in processes such as absorption and elimination of drugs, contributing, many times, to the multidrug resistance to chemotherapy [60]. These proteins are characterized by a 12-transmembrane domain (TMD) in the cellular membrane and two cytosolic sites for ATP-binding and hydrolysis [49].

In particular, the multidrug resistance protein 4 (MRP4) is a 160 kDa protein able to pump PGE_2 and other molecules (cAMP, cGMP, ADP, folic acid, etc.) out of the cell using the energy released from ATP hydrolysis [49, 60]. This specific member of the C subfamily of ABC transporters possesses the simplest structure of the ABC proteins and wider distribution across the body [61]. MRP4 is encoded by the highly polymorphic gene ATP-binding cassette sub-family C member 4 (*ABCC4)* and has been shown to contribute to angiogenesis, cell migration, and proliferation, playing a particular role in the development and prognosis of cancer [60, 62].

Once in the extracellular environment, prostanoids can bind to different G-protein coupled receptors, specific for each molecule, which, in turn, activate numerous pathways within the cell [46]. There are nine different highly selective receptors for prostanoids and PGE_2 is able to bind to EP1, EP2, EP3 (which are splice variants) and EP4, also known as PTGER1, PTGER2, PTGER3, and PTGER4, respectively [46, 55]. PGE_2 receptors are ubiquitously expressed in most organs and can potentially activate many cellular effects with oncogenic potential: apoptosis, cell proliferation, angiogenesis, invasion, immune surveillance, and inflammation [52, 63]. In fact, EP2 and EP4 have been associated with tumorigenic processes, with EP2 being overexpressed in cancers like esophageal, colorectal, and lung [55, 56].

1.4.3. PGE_2 catabolism

PG levels *in vivo* are determined not only by its synthesis but also by the degradation rates. PGE_2 catabolism depends on its internalization and inactivation, which are performed by two distinct proteins [46]. This PG is first transported into the cell via **prostaglandin transporter (PGT)**, also called organic anion transporting polypeptide 2A1 (OATP2A1), encoded by the solute carrier organic anion transporter family, member 2A1 *(SLCO2A1)* gene, and then oxidized by **15-hydroxyprostaglandin dehydrogenase (15-PGDH)**, encoded by the *HPGD* gene [46, 64]. Both of these proteins are essential for the efficient inactivation of oncogenic PGE_2, as supported by studies reporting that PGs were oxidized in a rapid manner only when PGT and 15-PGDH were co-expressed [46, 64].

PGT is a 12-TMD cell-surface transporter that mediates the uptake of prostanoids by the cells, functioning as a lactate/PG exchanger [50, 64]. In fact, the transport of PGE_2 into the cell by this protein is considered to be the rate-limiting step in this PG inactivation [64]. Movement into and out of the cells via PGT has been reported, however, most studies indicate the preferential movement of PGs from the extracellular fluid into the cell [50]. The process involving the intracellular compartmentalization of PGE_2 is not fully understood, but it is believed that the PGs coming from the extracellular environment are distributed to the compartments where 15-PGDH is located, whereas newly synthesized molecules locate in different locations within the cell [64]. The absence of PGT expression is related to the accumulation of PGE_2 in the extracellular milieu and, consequently, to a decrease in the conversion of this PG into its biologically inactive metabolite by 15-PGDH [50].

15-PGDH, a nicotinamide adenine dinucleotide (NAD^+)-dependent protein widely distributed across mammalian tissues, is one of the key enzymes in PG catabolism and its enzymatically active complex is formed by dimerization of two 29 kDa monomers [49, 53]. 15-PGDH is a physical antagonist of COX-2 and catalyzes the oxidation of the 15(*S*)-hydroxyl group of PGE_2 to yield a product, 15-keto-PGE_2, with reduced binding affinity to the EP receptors and, therefore, with reduced biological activity [53, 56]. Local conversion of PGE_2 to inactive metabolites is important to control its biologically-active levels due to the autocrine or paracrine nature of its action (near the site of synthesis) [50]. A few studies have reported decreased or even absent 15-PGDH expression in several cancers and NSAIDs have even been associated with *HPGD* upregulation in medullary thyroid and colorectal cancers [65-67].

1.4.4. The COX-2/PGE_2 pathway in GC

As mentioned above, the regular use of NSAIDs or COX-2 selective inhibitors (COXIBs) is associated with a decreased risk of some types of cancer, including GC, as well as with a decrease in the mortality rate of this disease [68]. This dose-dependent association is related to the suppression of the COX-2 enzymatic activity and, consequently, to lower levels of PGE_2, which plays an important role in gastrointestinal cancer development [69]. In specific, the role of aspirin in GC development has been explored in a population-based case-control study by Akre *et al.* [70]. In their published report, the authors found an association between the use of this NSAID in a Swedish population and a risk reduction for GC development, suggestively in a dose-dependent manner in both cardia and non-cardia GC [70]. This association was stronger for individuals with *H. pylori* infection, although with limited precision, which suggests a direct or indirect link between aspirin protection and gastric carcinogenesis associated with this

bacterium [70]. Nevertheless, as previously mentioned, the use of aspirin is associated with negative side effects, such as the risk of upper and lower gastrointestinal bleeding [71].

Due to the importance of PGE_2 in gastric carcinogenesis, some important aspects regarding its involvement in GC are reviewed below.

1.4.4.1. Expression and role of PGE_2 pathway in GC

The role of this prostanoid pathway, mainly of COX-2 and PGE_2, has been characterized throughout the years. The elevated levels of PGE_2, resulting from the upregulation of COX-2 and/or downregulation of 15-PGDH, and the role of the COX-2 pathway in carcinogenesis have been explored in a variety of cancers, such as colon [72], oral [73], breast [74], head and neck [75], pancreas [76, 77], and lung [78, 79]. Moreover, PGE_2 levels are inversely correlated with survival rates and this PG has been suggested as a prognostic biomarker of some cancers [80].

Regarding GC, most studies report similar findings: high PGE_2 levels and a role of the COX-2 pathway in gastric carcinogenesis [48, 81]. However, a study by Chen *et al.* [82] reported lower levels of PGE_2 in *H. pylori*-negative Han Chinese patients with low-grade intraepithelial neoplasia compared to *H. pylori*-positive patients, as well as lower levels in more severe stages of the disease. The authors attribute their results to the protective role of PGE_2 in the damaged gastric cells but do not compare their controversial results with other studies [82]. On the other hand, a case-control study by Dong *et al.* [83] in Shangai, China, aimed to find an association between the urinary PGE_2 metabolite (PGE-M) and risk for GC development, highlighting that higher concentrations of urinary PGE-M correlate with higher GC risk. The authors further suggested that this metabolite may serve as both a marker of underlying inflammation and a marker that reflects an upregulated COX-2 pathway associated with imminent development of cancer [83].

COX-2 is the most extensively studied protein of the COX-2/PGE_2 pathway. The expression of this enzyme has been frequently reported in many human tumors and it ranges from 10% in superficial gastritis to approximately 70% in GC, increasing throughout the progression of cancer, contrary to what happens with COX-1, whose levels remain constant [48, 84]. Ristimaki *et al.* [85] were the first to report the expression of COX-2 in GC, in 1997. They based their study in epidemiological studies which suggested that the use of aspirin reduced the incidence of gastrointestinal cancers and their mortality [85]. As the best-known target of aspirin and other NSAIDs, COX-2 expression was studied in human gastric carcinoma tissues using Northern blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), and immunohistochemistry [85]. Their results

showed, for the first time, a significantly higher level of COX-2 in human gastric adenocarcinoma tissues comparing to the paired tissues devoid of tumor [85].

Besides mediators like growth factors, proinflammatory cytokines, gastrin, and mitogen, *H. pylori* is related to the higher levels of COX-2 [86, 87]. Its infection has been shown to induce the expression of this enzyme, resulting in increased levels of PGE_2 in both malignant and pre-malignant lesions, and its eradication results in the suppression of COX-2 expression [86, 87]. Also, treatment of GC cells with tobacco components induced PGE_2 release mediated by COX-2, cyclin D1 expression, and cell proliferation, suggesting an association between smoking and gastric carcinogenesis [88]. Overall, COX-2 expression has been associated with intestinal-type GC, which may reflect distinct genetic alterations during carcinogenesis, large tumor size, proximal location, depth of invasion, and advanced clinical stage (noncurative operation) [48, 84, 87, 89, 90]. The expression of COX-2 has also been positively correlated with the expression of important molecules in angiogenesis, such as vascular endothelial growth factor (VEGF), suggesting its importance in that process [91-93]. In fact, some studies report significantly greater microvessel density in patients with overexpression of COX-2 [92]. A positive correlation has also been found between COX-2 expression and the expression of B-cell lymphoma 2 (BCL-2), an apoptosis inhibitor, and matrix metalloproteinase (MMP), involved in the degradation of the extracellular matrix of tumor cells, facilitating the diffusion of the tumor cells, the invasion of surrounding tissues, such as lymph nodes, and metastization [92]. The levels of transforming growth factor-beta (TGF-β) receptor type 2, involved in the regulation of apoptosis, are reduced by COX-2 expression, contributing even more to the inhibition of programmed cell death [92]. Therefore, COX-2 is often related to poor prognosis in patients with gastric malignancies [86, 87, 92]. This prognostic role has been explored by several studies and it appears to be more pronounced in lower stages of the disease, before the occurrence of major chromosomal changes in the tumor cells, allowing to reveal tumors with aggressive potential still in early stages [94, 95].

The expression of **15-PGDH**, COX-2 physiological antagonist, has also been studied in several types of cancer [96, 97]. It has been shown that its expression is higher in human normal tissue comparing to the paired cancerous tissues, such as breast, lung, stomach, kidney, pancreas, liver, small intestine, and colon [96, 97]. This downregulation has been detected together with COX-2 overexpression and, in fact, NSAIDs have been shown to upregulate 15-PGDH expression in cancers such as colorectal and medullary thyroid [65]. The overexpression of 15-PGDH in cancer cells such as those from glioma, breast, lung, and colon cancer led to the reduction of their proliferation, suggesting a contribution of *HPGD* expression to the inhibition of malignant phenotypes [53]. At a cellular level, immunoreactivity for 15-PGDH protein has been observed mainly in

noncancerous epithelium, namely in the cytoplasm of epithelial cells and in inflammatory cells in the lamina propria [98]. In cancer cells, immunoreactivity is usually weak or even absent, but it could be observed in the cytoplasm of some cells [98].

Regarding PGE_2 transporters, not much is known in GC. Nevertheless, in an *in vitro* study by Chen *et al.* [99], *ABCC4* knockdown using short hairpin ribonucleic acid (RNA) delivered by lentivirus blocked cell cycle progression in carcinogenesis, thus inhibiting the growth of the GC cells. Two other *in vitro* studies associated loss of *ABCC4* expression with higher cell sensitivity to chemotherapeutic drugs [61, 100]. Furthermore, *ABCC4* inhibition was also involved in the decrease of drug-resistant GC cells proliferation and tumor growth [61]. **MRP4** role in cancer involves not only the COX-2/PGE_2 pathway but also the cAMP-PKA pathway and the extrusion of toxic substances, which can both contribute to cancer progression [60]. In fact, most studies focus on the latter to explore the role of this transporter in a variety of cancers, such as leukemia [101], neuroblastoma [102, 103], pancreatic [104], ovarian [105, 106], and lung cancer [107]. Moreover, the copy number variation of *ABCC4* has been associated with esophageal squamous cell carcinoma [108]. Inhibition of this protein using RNA interference led to a decrease in the *in vitro* proliferation and colony formation in neuroblastoma, pancreatic, and gastric cancers [60]. *ABCC4* blockade also induced apoptosis in a xenograft acute myelocytic leukemia (AML) model and in colorectal carcinoma cells [60].

The prostaglandin transporter, **PGT**, is ubiquitously expressed in the human body [109]. At a protein level, it has been detected in the luminal membrane of the endothelial cells forming the blood-tumor and blood-brain barriers, in the glandular and luminal epithelium of the endometrium, and in the gastrointestinal tract, in specific in the parietal cells and in the pyloric glands of the gastric antrum [110, 111]. PGT expression remains controversial among different types of cancer. High expression has been detected in the liver [112], breast [113], and bone tumors [111]. Moreover, PGT upregulation has been found in primary and metastatic liver cancer [111], pancreatic adenocarcinoma [114], and prostate cancer metastasis [115] compared with normal tissues. On the other hand, reduced expression has been found in cancers such as small [116] and non-small cell lung cancer [115], kidney, and colorectal cancer [109, 113], appearing to have a role in the regulation of the extracellular levels of PGE_2 [111]. Furthermore, an inactivating mutation on the *SLCO2A1* gene has been associated with colon cancer and also with resistance to NSAIDs [117].

In triple-negative breast cancer, a high COX-2, high-MRP4, low 15-PGDH, and low-PGT expression pattern has been detected and associated with the tumor-promoting PGE_2 high levels in the tumor microenvironment, contributing to the overall poor prognosis of this type of cancer [118].

1.5. Genetic susceptibility

The exposition to a given carcinogen will only result in the development of cancer in a portion of the exposed individuals, suggesting that there are susceptibility differences in the carcinogenic process that can affect the risk of a person to develop cancer [119]. As previously mentioned, this susceptibility can be attributed to inherited genomic variations, such as SNPs, which modulate the risk of cancer in a synergistic manner [120]. SNPs are the most abundant type of variation in the human genome, being found, by definition, in more than 1% of the world population, otherwise it is classified as a rare mutation [121, 122]. It consists of a single position in the genome that varies at polymorphic levels and it is usually biallelic, i.e., the SNP site may be occupied by one of two distinct bases [121]. Most SNPs occur in noncoding regions, affecting rates of transcription when present in regulatory sites, for example [121]. In coding regions, these polymorphisms may affect protein structures and consequent functions, which may result in disease development or drug resistance [121]. In such regions, the SNPs that alter the predicted amino acid are termed nonsynonymous, whereas the ones that are silent are known as synonymous [123]. The understanding of genome sequence variation, from SNPs to large copy variants, has provided a step forward towards the elucidation of the environmental/pathogenic impact on the genome, the genetic history across populations, and the heterogeneous distribution of cancers [123].

Linkage disequilibrium (LD) can be defined as the nonrandom association of alleles at different loci and estimates the correlation between SNPs [123]. Therefore, SNPs inherited together are in strong LD and, in that case, one single SNP can be tested and provide information for all correlated polymorphisms [123]. The **GWAS** are experimental designs used to study associations between traits and genetic variants and rely on LD [124]. The primary goal of this type of study is the understanding of the disease biology and, over the last decade, it has allowed the discovery of SNPs associated with increased risk of many diseases, namely cancer [120, 124]. GWAS involve large cohorts of individuals and the analysis of hundreds of thousands of variants across their genome in order to identify variants associated with a given trait [125]. The strategy is based on testing tagSNPs, SNPs in a region of the genome with high LD (high correlation, $r^2>0.8$) that represent a haplotype, highlighting regions with susceptibility alleles and avoiding the expensive sequencing of all existing SNPs [123, 126]. To date, there are seven GWAS of gastric adenocarcinoma and, of those, only one was not conducted in East Asian ancestry populations, which translates the high incidence of this type of cancer in that region [127]. Those studies reported 11 risk loci, with one risk loci and one risk haplotype identified in Icelandic GC cases [127]. Furthermore, most studies published before 2015 followed a

candidate gene approach, with almost 50% focusing in the immune/inflammatory landscape or adhesion/invasiveness [31].

1.5.1. Genetic susceptibility and the PGE_2/COX-2 pathway

Genetic variability in COX-2/PGE_2 pathway has been explored throughout the years, especially in colorectal cancer [128-130]. Our group has previously explored the role of tagSNPs in *PTGS2*, *HPGD*, *SLCO2A1*, and *ABCC4* genes in colorectal cancer, in early stages of tumor development, as well as in the recurrence of colorectal adenomatous polyps in a Northern Portuguese population [128, 131].

Genetic polymorphisms in the gene encoding COX-2 are associated with increased cancer risk in a variety of sites and have been extensively studied (reviewed in [132]). Regarding 15-PGDH, genetic variants of the *HPGD* gene have been associated with colorectal, prostate, and breast cancer susceptibility [129, 133-135].

Not much is known about the effects of genetic variability in *ABCC4* and *SLCO2A1* genes in cancer, but the *ABCC4* SNP rs1729786 has been associated with survival of patients with low-stage non-small cell lung cancer [136].

Given the importance of genetic susceptibility studies and the pivotal role of the PGE_2 pathway in a variety of cancers, the understanding of the four mentioned proteins is fundamental, especially considering the lack of information in Caucasian patients.

2. AIMS OF THE STUDY

Considering there are molecular and genetic differences between ethnic populations and that most studies focus on Asian GC patients, the main purpose of this thesis was to assess the role of the PGE_2 pathway, namely COX-2, MRP4, 15-PGDH, and PGT, in the development of GC in a Caucasian population, with the following specific aims:

1. To characterize the genomic profile of PGE_2 pathway associated with GC development in a Caucasian population by targeting the *PTGS2, ABCC4, HPGD,* and *SLCO2A1* genes (**Study 1**);

2. To explore the genetic expression of *PTGS2, ABCC4, HPGD,* and *SLCO2A1* genes in GC (**Study 2**);

3. Additionally, we further aimed to evaluate the influence of the genetic polymorphisms highlighted in Study 1 on the mRNA expression of *PTGS2, ABCC4, HPGD*, and *SLCO2A1* genes characterized in Study 2.

3. MATERIAL AND METHODS

3.1. Study design

This research project was approved by the Ethics Committee at the *Instituto Português de Oncologia do Porto* (IPO-Porto) (CES.314/016).

3.1.1. Sample Size Estimation

To achieve a statistical power of 80%, with a significance level of 5%, for polymorphisms with a frequency superior to 15%, we estimated that the sample size required to detect an Odds Ratio (OR) equal or superior to 1.70 is 200 patients and 400 controls, i.e., a 2:1 ratio (Epi Info version 6, Centers for Disease Control and Prevention, Atlanta, Georgia). Considering a r^2, which is used to select the tagSNPs and is inversely related to the magnitude by which the sample size must be increased in a study design, of 0.8, we had to increase our sample size by 25%, resulting in a total of 750 expected participants (500 controls and 250 GC patients).

3.1.2. Study population

A **non-matched hospital-based case-control study** was designed that included 736 participants: 476 cancer-free controls and 260 histologically confirmed intestinal-type GC patients. All from the Northern region of Portugal and recruited at IPO-Porto.

In the **control group**, individuals without clinical evidence of CG or any other malignancy with or above 50 years old were included. These individuals were randomly recruited from the service of blood donation at IPO-Porto between July 2005 and February 2008 and integrated a DNA database of over one thousand blood donors.

The **GC patients group** gathered participants with age equal or superior to 50 years old with histological confirmation of intestinal-type GC between May 2012 and December 2015. These patients were consecutively selected after reviewing the histopathological database from the Pathology department at IPO-Porto, based on the availability of formalin-fixed paraffin-embedded (FFPE) samples.

Medical records were reviewed to extract the clinicopathological variables, such as localization, stage, and tumor grade. All tumors were restaged according to the eighth edition of the AJCC Cancer Staging Manual [44]. For the purpose of this thesis, we focused on the pathological staging (pTNM).

3.2. Sample collection and processing

For each patient, whenever possible, six 10 µm sections of FFPE sample were obtained based on the most representative 3 µm section previously stained with hematoxylin and eosin (H&E), stored at the Pathology department at IPO-Porto. The selected section was further histopathologically characterized by a pathologist and the tumoral and normal-appearing mucosa area, distant to the tumor whenever feasible, delimited.

3.2.1. Nucleic acid extraction

Both DNA and RNA were extracted from FFPE tissue using the AllPrep® DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Besides its yield and quality, this kit was chosen because it was the only one able to simultaneously extract both RNA and DNA from the same FFPE sample, allowing us to save time and sample. Moreover, Patel *et al.* [137] selected AllPrep® DNA/RNA FFPE kit as the most appropriate for FFPE tissue from a panel of 14 commercially available kits. The number of 10 µm sections used for nucleic acid extraction varied from two to six, depending on the size of the limited area, enriched in "normal" or tumor cells (up to 6 cm^2). Briefly, using a sterile single-use scalpel, the area was macrodissected into a 1.5 mL microcentrifuge tube containing 1 mL of deparaffination reagent D-limonene (Santa Cruz, Dallas, TX, USA) by scratching. This reagent was recommended as a more environmental-friendly solution comparing to xylene, having similar nucleic acid yields and qPCR quality [138]. The kit instructions were followed throughout the remaining procedure.

The resulting DNA and RNA were quantified using the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and their quality was assessed by measuring the optical density (OD) 260/280 ratio. We obtained a mean DNA and RNA concentration of 65.3 ng/µL and 475.9 ng/µL and a mean 260/280 ratio of and 1.81 and 1.95, respectively. DNA was kept at -20°C until genotype assessment.

RNA quality can be indicated by RNA integrity number (RIN) scores obtained from microcapillary electrophoresis, an algorithm able to attribute integrity values to purified RNA [139]. Despite its usefulness for other types of tissue, chemical modification of RNA induced by formalin is not resolved by electrophoresis and Groelz *et al.* [139] concluded that this algorithm cannot predict the performance of RNA from formalin-fixed tissue in downstream applications, so we did not proceed with this assessment [140].

3.3. Characterization of the genomic profile of PGE_2 pathway associated with GC development by targeting the *PTGS2, ABCC4, HPGD,* and *SLCO2A1* genes (Study 1)

3.3.1. Genetic variants selection

The method for polymorphism selection was previously described by our group in 2014 [128]. Briefly, using the Genome Variation Server (version 7.00) from the Seattle SNP database, the genetic variants were retrieved from a set of common SNPs in the Caucasian population from HapMap project (CEU), using a tagSNP approach. TagSNPs were retrieved with a minor allele frequency equal or superior to 15%, within the coding region of the gene plus 2 kb upstream and downstream, and with a r^2 superior to 0.8. In total, 140 tagSNPs were captured: 6 in the *PTGS2* gene, 15 in *HPGD*, 88 in *ABCC4*, and 31 in *SLCO2A1*. The Sequenom platform (Sequenom, San Diego, CA, USA) was used to further select the SNPs with high likelihood of genotyping success, resulting in a total of 55 SNPs successfully converted to the platform. Briefly, prioritization was as follows: first, all non-singletons tagSNPs or singletons with expected functional repercussion (FuncPred software) were tested. TagSNPs with low genotyping scores were replaced with representative variants and, finally, the non-significant singletons were included in the design of the array.

Furthermore, the rs5275 and rs689466 polymorphisms in *PTGS2* gene, that were previously associated with tumor development and failed to be converted to the Sequenom, were also included.

3.3.2. DNA purification

Since water-diluted DNA was recommended for the genotyping step described below and the AllPrep® DNA/RNA FFPE kit yielded buffer ATE-diluted DNA, the samples were further purified using GRS Pure DNA Kit (Grisp, Porto, Portugal). Briefly, DNA Binding Buffer, which allows easy binding of the DNA to the glass fiber matrix of the spin column, was added to the sample. Then, in a simple centrifugation step, contaminants, such as proteins, unincorporated nucleotides, divalent ions, and enzyme inhibitors, were completely removed using an ethanol-containing Wash Buffer. Finally, purified DNA was eluted by a low salt Elution Buffer that consisted of 10 mM Tris-HCl pH 8.5.

3.3.3. Polymorphisms characterization

The aim of genotype characterization in disease genetics is to identify polymorphisms that are related to changes in cellular biological processes [121]. Despite the existence of many genotyping technologies available, SNP characterization is in constant development and there is not a single platform or technology that can satisfy all study designs [141]. In this study, tagSNP genotyping was performed using MassARRAY® iPLEX Gold Technology (Agena Bioscience, San Diego, CA, USA), which is based on multiplex amplification followed by mass-spectrometric product separation (**Figure 7**). DNA samples are amplified by PCR using gene-specific primers and an iPLEX single base extension is performed to identify the locus-specific alleles, which will have distinct masses. The data analysis software can then differentiate between SNP alleles based on that mass difference. This technology uses a single termination mix and universal reaction conditions for all SNPs, small reagent volumes and allows the assessment of up to 40 SNPs per sample. This technique was carried-out by *Centro Nacional de Genotipado* (CEGEN) in *Santiago de Compostela*, Spain, and a total of 250 samples were sent, based on the quantity of DNA. Of those, 241 samples were considered of good quality and successfully genotyped.

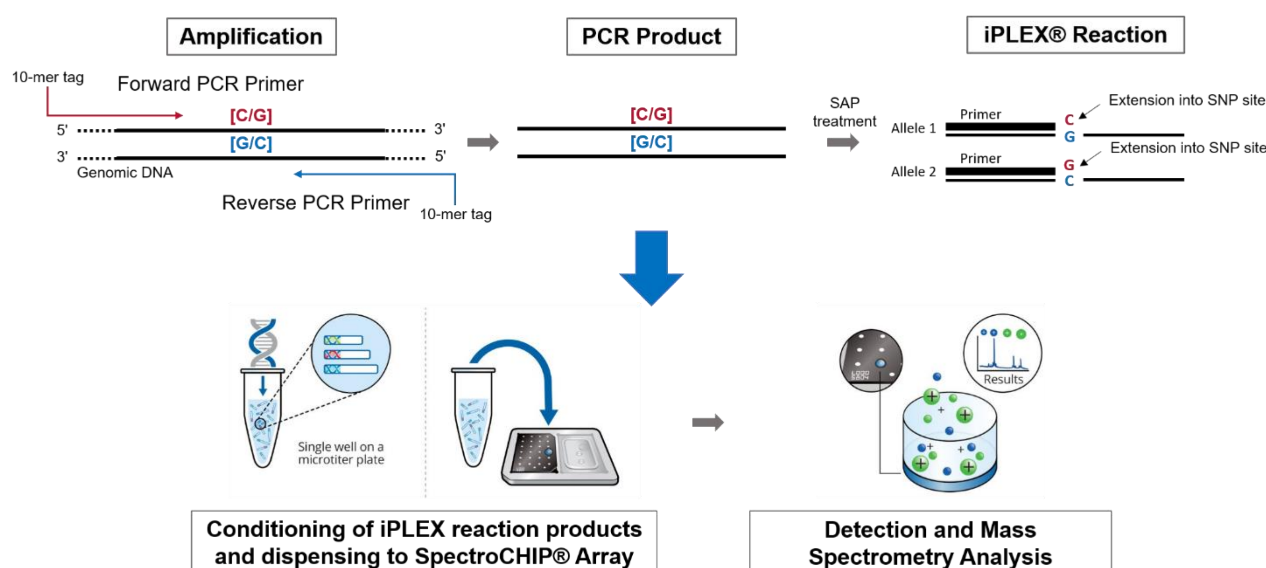

Figure 7. Overview of genotyping workflow. The region of interest undergoes amplification up to 40 fragments in a single reaction and the PCR products are treated with Shrimp Alkaline Phosphatase (SAP) to neutralize dNTPs that have not been incorporated. The iPLEX® reaction is performed to identify the locus-specific alleles and its cocktail includes buffer, primers, enzyme, and terminator nucleotides. Next, a small amount of sample is transferred to a single pad on the SpectroCHIP® Array and, finally, hundreds of mutations can be tested per sample by mass spectrometry. Adapted from http://agenabio.com/.

The polymorphisms not included in this tagSNP analysis, the rs5275 and rs689466 in *PTGS2* gene, were characterized in 198 GC patients with available DNA sample through allelic discrimination using the validated TaqMan® SNP genotyping assays (Thermo Fisher Scientific, Waltham, MA, USA) C__7550203_10 and C__2517145_20, respectively, by Real-Time PCR.

Genotypes were excluded from the analysis by the following criteria: call rate inferior to 0.90, concordance rate inferior to 0.95, and Hardy-Weinberg equilibrium (HWE) with P<0.05 in the control population. Two no-template controls and three positive controls, representing each genotype, were included in the 96-well plates used to characterize the rs5275 and rs689466 genetic variants. Ten percent of the samples were resubmitted to a new genetic characterization by random selection to confirm the results.

3.3.4. Statistical analysis

Data analysis was performed using the computer software *IBM® Statistical Package for the Social Sciences (SPSS®) Statistics* (IBM Corp., Armonk, NY, USA) version 26.0 for Windows. Categorical variables were compared using chi-square (X^2) analysis with a 5% level of significance and nonparametric (Mann-Whitney) tests were used to compare mean values.

The Hardy-Weinberg principle states that, in the absence of disturbing factors, the genetic variation in a population will remain constant from generation to generation [142, 143]. The HWE was thus tested by the Pearson's goodness of fit test to compare the genotype frequencies observed versus the expected. If P value was inferior to 0.05, control genotype distributions were assumed to deviate from HWE.

OR and its confidence interval (CI) were estimated by a multivariate logistic regression analysis as a measure of association between the polymorphisms and the risk for GC development. Age and sex were included as covariates in this analysis and homozygotes for the allele with the highest frequency were defined has the reference genotype for OR estimation. We tested the following models of inheritance: codominant, dominant, recessive, and overdominant.

For the haplotype analysis performed at a gene level, the implementation of the expectation-maximization (EM) algorithm coded into the *haplo.stats* package was used to estimate haplotype frequencies. The reference group was automatically selected and corresponded to the most frequent haplotype and the haplotype blocks were defined considering the most meaningful SNPs. Both logistic regression and haplotype analysis were performed using the SNPStats software (https://www.snpstats.net/start .htm) [144].

Kaplan-Meier analysis was performed using a log-rank statistical test to assess the correlation between the genetic polymorphisms and the age at diagnosis.

The “gene-environment” interactions in GC development were assessed by applying a nonparametric approach using the open-source multifactor dimensionality reduction (MDR) software (version 3.0.2) (https://www.multifactordimensionalityreduction.org/). The competence of an MDR model is evaluated by its testing accuracy and cross-validation consistency (CVC) and, in general, the single best one reaches the maximum of these two parameters. Using 10-fold cross-validation (CV), a technique used to estimate the error of a predictive model, the data were divided into ten parts, in which nine are known as the training data and used to develop the method and one corresponds to the independent testing data. The CVC is, thus, a measure of the number of times (up to ten) the best model was chosen [145]. Statistical significance was determined using a 1000-fold permutation test, where the case and control labels were randomized 1000 times in the original dataset to create permuted datasets, in which the MDR was run to determine the expected distribution of testing accuracies under the null hypothesis.

The false discovery rate (FDR) is the expected proportion of false discoveries (type I errors) in a set of results and was used to correct for multiple testing and confirm the noteworthiness of significant findings [146, 147]. This was performed by running the following syntax in SPSS software and the significant results presented a test value equal to 1:

```
DATA LIST free / p (F5.3).
BEGIN DATA
,021 ,022 ,027 ,19 ,007 ,57 , and all the other P values
END DATA.
SORT CASES by p (a).
COMPUTE i=$casenum.
SORT CASES by i (d).
COMPUTE q=.05.
COMPUTE m=max(i,lag(m)).
COMPUTE crit=q*i/m.
COMPUTE test=(p le crit).
COMPUTE test=max(test,lag(test)).
FORMATS i m test(f8.0) q (f8.2) crit(f8.6).
VALUE LABELS test 1 'Significant' 0 'Not Significant'.
LIST.
```

To determine if the genetic variants identified as susceptibility biomarkers for GC in this study could be determinants of mRNA expression, we performed one-way ANOVA to compare the mean tissue expression between the three possible genotypes and

student's t or nonparametric tests when appropriate to assess the mean tissue expression between two genotypes or models of inheritance. The assessment of *PTGS2, ABCC4, HPGD,* and *SLCO2A1* mRNA expression in GC is explained in the following study.

3.4. Assessment of the *PTGS2, ABCC4, HPGD,* and *SLCO2A1* mRNA expression in GC (Study 2)

3.4.1. cDNA synthesis (reverse transcription)

A subset of the GC group was randomly selected, including approximately one hundred samples of "normal"-appearing mucosa and one hundred samples of tumoral mucosa.

Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a very powerful tool for the detection and quantification of mRNA due to the exponential generation of DNA template copies [148, 149]. Moreover, real-time PCR allows the collection of data throughout the PCR run as it occurs, combining DNA amplification and detection into one single step [150].

For cDNA synthesis, oligo-dT priming should be avoided when using FFPE-derived RNA [140], so cDNA was synthesized from up to 2 µg of RNA using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, in a 20 µL reaction mix, 2.0 µL of 10X RT Buffer, 0.8 µL of 25X dNTP Mix (100 mM), 2.0 µL of 10X RT Random Primers, 1.0 µL of MultiScribe Reverse Transcriptase, 4.2 µL of nuclease-free water, and 10.0 µL of RNA were used. The RT conditions were as follows: annealing at 25 °C for 10 minutes, DNA polymerization at 37 °C for 120 minutes, and enzyme deactivation at 85 °C for 5 minutes.

All RT reactions included one no-template negative control. Moreover, 1 µL containing 1 µg of the QPCR Human Reference Total RNA, part of the Absolutely RNA FFPE Kit (Agilent, Santa Clara, CA, USA), was used as a positive control to monitor the quality of the RT.

3.4.2. Gene expression assays selection and validation

Studies have shown that FFPE-resulting RNA fragments are large enough to be detected by TaqMan® technology, which has higher specificity and reproducibility than SYBR® Green, as long as the amplicons are short [151]. Nevertheless, considering the RNA damage across the length of this type of sample, the selection of TaqMan® Gene

Expression assays needs to be thorough. Thus, we took into consideration the following features:

1) **Size** – as small as possible because there is a direct correlation between the size of the target amplicon and the number of intact target templates available;
2) **Coverage** – the best possible, preferably spanning exons;
3) **Number of citations**;
4) **Genomic DNA detection** – considered a disadvantage.

All TaqMan® assays were further validated to determine the efficiency of the amplification reaction and their limit of detection using a 1:2 dilution series with 7 dilution steps, starting with 200 ng of cDNA input. Efficiency between 90% and 105% and sensitivity above 6.25 ng were reported for all gene expression assays used.

3.4.3. Reference gene selection

Although Real-Time PCR is widely used to quantify mRNA expression, some problems are associated with its use, including distinct RT and PCR efficiencies, inherent variability of RNA, and variability of extraction protocols [152]. Therefore, it is important to select an accurate normalization method and the selection of an internal reference gene is the most common approach [153].

A panel of six reference genes was selected to determine the most adequate for this experiment based on the available literature on gastrointestinal cancers [154, 155]. The gene expression of *B2M*, *HPRT1, RPL29*, *PPIA*, *IPO8*, and *GUSB* was assessed using the Hs00187842_m1, Hs02800695_m1, Hs00988959_gH, Hs99999904_m1, Hs00914057_m1, and Hs99999908_m1 TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA), respectively.

With the development of statistical algorithms that allow the selection of the most stable reference gene, such as NormFinder, geNorm, and BestKeeper, the use of multiple housekeeping genes was proposed [156]. We used the first two softwares to select the most suitable gene to normalize our results and both assess the expression stability (*M*) of each candidate gene – lower *M* values correspond to higher levels of expression stability [157, 158]. Additionally, NormFinder takes into account intra-and intergroup variations, providing the best combination of two candidate genes [156].

In total, 13 tumor samples and 11 "normal"-appearing mucosa samples from stomach were used to determine the most stable gene across GC and normal gastric tissue (GN). The *M* values are presented in **Table 3.** The geNorm algorithm selected *HPRT1* as the most stable gene, with a stability value equal to 0.541. On the other hand,

the NormFinder algorithm determined that the best endogenous control was *IPO8*, with a stability value of 0.106. Furthermore, the best combination of two genes provided by this software was *HPRT1* and *IPO8*, with a stability value of 0.094. Thus, these two genes were selected as the most suitable reference genes for this study. *HPRT1* codifies the protein hypoxanthine phosphoribosyl transferase (HPRT), which uses preformed purine bases and phosphoribosyl pyrophosphate (PRPP) as substrates to catalyze the formation of nucleotide monophosphates [159]. *IPO8* has only been suggested as a potential reference gene a little over a decade ago and encodes a member of importin β family, IPO8, involved in nuclear import [160, 161]

Table 3. Stability expression values for normalization of GC and GN samples from NormFinder and GeNorm softwares.

Gene name	Algorithm	Intragroup variation (*M*)		Intergroup variation (*M*)
		GC	GN	
B2M	NormFinder	0.140	0.234	0.267
	GeNorm	0.648	0.799	0.761
HPRT1	NormFinder	0.027	0.060	0.125
	GeNorm	0.492	0.601	**0.541**
RPL29	NormFinder	0.098	0.045	0.244
	GeNorm	0.595	0.584	0.647
PPIA	NormFinder	0.044	0.099	0.451
	GeNorm	0.522	0.627	0.837
IPO8	NormFinder	0.032	0.101	**0.106**
	GeNorm	0.496	0.634	0.560
GUSB	NormFinder	0.146	0.118	0.185
	GeNorm	0.661	0.678	0.687

GC: tumorous mucosa samples; GN: normal mucosa samples
Values in **bold** correspond to the lowest *M* values for each algorithm.

3.4.4. Real-Time PCR

cDNA amplification by Real-Time PCR was performed using a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). In a 10 µL reaction mix, 5.0 µL of TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µL of TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA), and 20 ng of cDNA template were used.

The gene expression assays used to measure the mRNA expression of the *PTGS2*, *HPGD*, *ABCC4*, and *SLCO2A1* genes were Hs00153133_m1, Hs00168359_m1, Hs00988717_m1, and Hs01114926_m1 (Applied Biosystems, Foster City, CA, USA), respectively. All assays underwent the following thermal cycling conditions: 50° C for 2 minutes, 95° C for 10 minutes, and 45 cycles of 95° C for 15 seconds and 60° C for 1 minute.

For mRNA quantification using real-time PCR, triplicates were used and replicates with a standard deviation (SD) superior to 30% of 1 C_T were excluded. All the target and reference genes were included in the same plate for each sample, as illustrated in **Figure 8**. One positive control from the RT reaction and three no template negative controls were included. Both cDNA synthesis kit and TaqMan® Gene Expression Master Mix were recommended in an article by Thermo Fisher Scientific [162]. The endpoint of the real-time PCR was the cycle threshold (C_T) determined as the average value from three independent reactions. Regarding reference genes, we calculated the mean value between the C_Ts correspondent to *IPO8* and *HPRT1*.

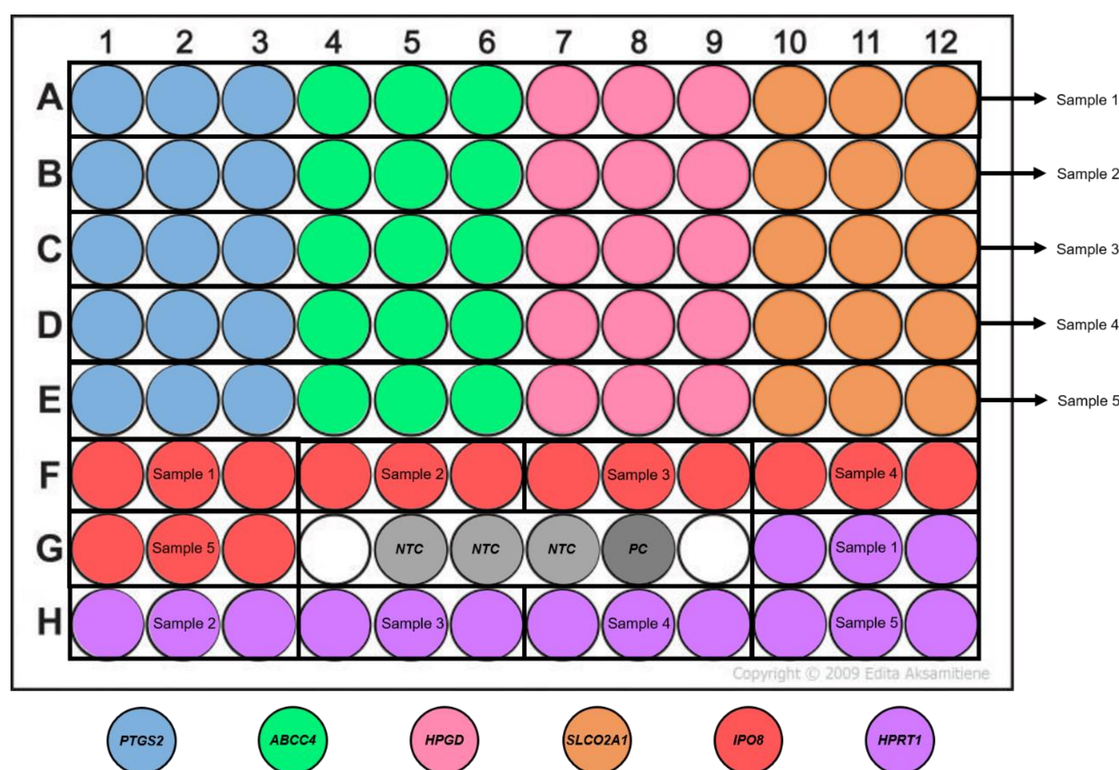

Figure 8. Plate design for mRNA expression analysis by real-time PCR. NTC: no template control; PC: positive control.

3.4.5. Statistical analysis

The relative mRNA expression was expressed as the difference between C_Ts correspondent to the amplification curves of the target genes (*PTGS2, ABCC4, HPGD, and SLCO2A1*) and the reference genes ($-\Delta C_T$). The expression fold-change was calculated following the Livak method ($2^{-\Delta\Delta Ct}$) [163].

Statistical analysis was performed using the computer software *IBM® SPSS® Statistics* (IBM Corp., Armonk, NY, USA) version 26.0 for Windows. Additionally, GraphPad Prism version 8.00 for Windows was used to obtain graphical designs. Student's t-test was performed to compare mean values between values (normal vs tumor, female vs male, stage I and II vs stage III and IV) and the correspondent nonparametric tests were applied when appropriate. Values were considered statistically significant at $P<0.05$.

4. RESULTS

4.1. Description of the study population

The characterization of the study population is summarized in **Table 4.** Cases were significantly older than controls (median age of 70 vs 58, respectively, P<0.001). Males were overrepresented in both groups, particularly in the group of controls, although not with statistical significance (58.5% and 65.5% in cases and controls, respectively, P=0.057). Most tumors were located in the antrum and corpus-antrum transition (61.6%) and presented moderately differentiated cells (60.6%). Regarding tumor staging, nearly 60% of GC patients were diagnosed in stages I and II (56.0% vs 44.0% for stages III and IV). An enrichment towards early stages was noticed when comparing to the proportions reported in the IPO-Porto oncologic registry published in 2017 (49.5%, P=0.044) [164].

Table 4. Description of participants.

	Cases (n=260)	Controls (n=476)	P value
Demographics			
Age (years)			
Mean±SD	69.87±0.60	57.98±0.23	
Median (min-max)	70 (50-92)	58 (50-69)	<0.001
Sex, n (%)			
Male	152 (58.5)	312 (65.5)	
Female	108 (41.5)	164 (34.5)	=0.057
Tumor characteristics			
Tumor location, n (%)			
Cardia and GEJ	24 (9.4)	--	
Fundus and corpus	41 (16.1)	--	
Antrum and corpus-antrum transition	157 (61.6)	--	
Angularis incisura	7 (2.7)		
Others*	26 (10.2)		
Grade, n (%)			
Well differentiated	28 (10.8)	--	
Moderately differentiated	157 (60.6)	--	
Poorly differentiated	63 (24.3)	--	
Cannot be assessed	11 (4.2)	--	
Stage, n (%)			
I-II	145 (56.0)	--	
III-IV	114 (44.0)	--	
Synchronous tumors, n (%)			
Yes	6 (2.3)	--	
No	254 (97.7)	--	

SD: standard deviation; GEJ: gastroesophageal junction.
*Including tumors that occupy more than one location and tumors of the gastric stump.
For synchronous tumors, the most advanced lesion was considered in the characterization.

4.2. Characterization of the genomic profile of PGE_2 pathway associated with GC development by targeting the *PTGS2, ABCC4, HPGD,* and *SLCO2A1* genes (Study 1)

4.2.1. Genotype frequencies and risk estimates

The description of selected SNPs is summarized in **Table S1.** Nine SNPs were excluded, five due to genotyping failure and the other four due to deviation from HWE (P<0.05). Thus, a total of 48 SNPs were included in this analysis. The concordance rates were 100% for all genetic polymorphisms and the mean genotype call rate was 99.86%. Overall, eight genetic polymorphisms were implicated in the susceptibility for GC development, as displayed in **Table 5.** The risk estimates for the involvement of all analyzed genetic variants in GC onset are summarized in **Table S2**.

Homozygous individuals for the minor G allele of the rs689466 polymorphism in the *PTGS2* gene were overrepresented in the GC patients group (7.1% vs 3.4% in control group), leading to a 3-fold increase of GC risk (aOR=2.98; 95% CI: 1.14-7.74; P=0.027) in the multivariate analysis, including age and sex as covariates. Moreover, the Kaplan-Meier analysis showed that the estimated age at diagnosis is three years anticipated for these individuals compared with the ones carrying the A allele (70 vs 73 years; 95% CI: 71.48-74.52 and 62.49-77.52, respectively; P=0.011).

Following a recessive model, the rs1678374 and rs1678405 polymorphisms in the *ABCC4* gene were associated with a 51% protection for GC development in homozygous carrying the C allele (aOR=0.49; 95% CI: 0.26-0.91; P=0.019 and aOR=0.49; 95% CI: 0.23-1.03; P=0.049, respectively). Additionally, for the rs1751031 polymorphism in the same gene and following a dominant model, carriers of the minor allele were also associated with a 40% protection (aOR=0.60; 95% CI: 0.39-0.94; P=0.022).

Regarding the *HPGD* gene, the rs2303520 polymorphism was associated with a 65% increased risk for GC onset for carriers of GA genotype compared to homozygous individuals, following the overdominant model of inheritance (aOR=1.65; 95% CI: 1.05-2.59; P=0.031).

Three SNPs in the *SLCO2A1* gene showed an influence in GC susceptibility. Carriers of the rs11915399T allele presented a 38% decreased risk for this type of cancer (OR=0.62; 95% CI: 0.39-0.99; P=0.043), whereas both the rs10935090 and rs9821091 tagSNPs were associated with an increased risk in individuals carrying the homozygous minor allele genotype, with the former reaching over 4-fold enhanced susceptibility (OR=4.30; 95% CI: 1.22-2.53; P=0.026 and OR=1.95; 95% CI: 1.12-3.40; P=0.02, respectively). Furthermore, carriers of the TT genotype in rs10935090 genetic variation

showed a statistically significant ten-year anticipation in the estimated age at diagnosis compared to individuals carrying the C allele (62 vs 72 years; 95% CI: 59.61-64.39 and 71.81-74.19, respectively; P<0.001). A statistically significant early diagnosis was also observed for the AA genotype carriers of rs9821091 tagSNP following a recessive model (72 vs 71 years; 95% CI: 70.76-73.24 and 68.17-73.83, respectively; P=0.017). On the other hand, for the same genetic variant, the heterozygous individuals were diagnosed with GC two years later (73 vs 71 years in homozygous individuals; 95% CI: 69.06-72.94 and 71.43-74.57, respectively; P=0.018), even though its association with GC protection is not statistically significant (aOR=0.70; 95% CI: 0.46-1.05; P= 0.085).

Despite not being identified as susceptibility biomarkers for GC development, the time-to-age diagnosis analysis showed that the rs2555632 tagSNP in *HPGD* gene and the rs4241362 genetic polymorphism in *SLCO2A1* gene are linked to an anticipation in the age of diagnosis by two and seven years, respectively, in CC homozygous individuals compared to carriers of the T allele, as displayed in Table S2 (70 vs 72 years; 95% CI: 66.67-73.33 and 70.73-73.27, respectively; P=0.027, and 65 vs 72 years; 95% CI: 62.94-67.06 and 70.87-73.13; P=0.024, respectively).

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Table 5. Genotype frequencies among gastric cancer cases and controls, risk estimates for the involvement of *PTGS2, ABCC4, HPGD, and SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype frequencies		Univariate analysis			Multivariate analysis			Age at diagnosis		
		Cases, *n* (%)	Controls, *n* (%)	OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
PTGS2												
rs689466	Codominant											
	AA	121 (61.1)	322 (68.8)	1.00	-	0.054	1.00	-	**0.021**	73.00	71.25-74.75	-
	AG	63 (31.8)	130 (27.8)	1.29	0.89-1.86		1.50	0.93-2.42		73.00	68.89-77.11	0.21
	GG	14 (7.1)	16 (3.4)	**2.33**	**1.10-4.92**		**3.40**	**1.29-8.97**		**70.00**	**62.49-77.52**	**0.008**
	Dominant											
	AA	121 (61.1)	322 (68.8)	1.00	-	0.056	1.00	-	**0.022**	73.00	71.25-74.75	-
	AG-GG	77 (38.9)	146 (31.2)	1.40	0.99-1.98		**1.69**	**1.08-2.65**		73.00	69.94-76.06	0.058
	Recessive											
	AA-AG	184 (92.9)	452 (96.6)	1.00	-	**0.046**	1.00	-	**0.027**	73.00	71.48-74.52	-
	GG	14 (7.1)	16 (3.4)	**2.15**	**1.03-4.49**		**2.98**	**1.14-7.74**		**70.00**	**62.49-77.52**	**0.011**
	Overdominant											
	AA-GG	135 (68.2)	338 (72.2)	1.00	-	0.30	1.00	-	0.19	73.00	71.46-74.54	-
	AG	63 (31.8)	130 (27.8)	1.21	0.85-1.74		1.37	0.86-2.19		73.00	68.89-77.11	0.38
	Log-additive	-		**1.40**	**1.06-1.86**	**0.021**	**1.66**	**1.15-2.40**	**0.007**	-		
ABCC4												
rs1678374	Codominant											
	TT	90 (40.5)	161 (33.9)	1.00	-	0.076	1.00	-	0.063	72.00	69.48-74.52	-
	TC	107 (48.2)	234 (49.3)	0.82	0.58-1.15		1.04	0.67-1.63		72.00	69.74-74.26	0.47
	CC	25 (11.3)	80 (16.8)	**0.56**	**0.33-0.94**		**0.50**	**0.26-0.97**		73.00	71.00-75.00	0.57
	Dominant											
	TT	90 (40.5)	161 (33.9)	1.00	-	0.09	1.00	-	0.55	72.00	69.48-74.52	-
	TC-CC	132 (59.5)	314 (66.1)	0.75	0.54-1.04		0.88	0.58-1.34		72.00	70.77-73.23	0.66
	Recessive											
	TT-TC	197 (88.7)	395 (83.2)	1.00	-	0.05	1.00	-	**0.019**	72.00	70.38-73.63	-
	CC	25 (11.3)	80 (16.8)	0.63	0.39-1.01		**0.49**	**0.26-0.91**		73.00	71.00-75.00	0.39
	Overdominant											
	TT-CC	115 (51.8)	241 (50.7)	1.00	-	0.79	1.00	-	0.28	72.00	70.46-73.54	-
	TC	107 (48.2)	234 (49.3)	0.96	0.70-1.32		1.25	0.83-1.89		72.00	69.74-74.26	0.31
	Log-additive	-		**0.77**	**0.60-0.97**	**0.027**	0.78	0.58-1.06	0.11	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: Confidence Interval. Values in **bold** are statistically significant ($P<0.05$).

Table 5 (cont.). Genotype frequencies among gastric cancer cases and controls, risk estimates for the involvement of *PTGS2, ABCC4, HPGD, and SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype frequencies		Univariate analysis			Multivariate analysis			Age at diagnosis		
		Cases, *n* (%)	Controls, *n* (%)	OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs1678405	Codominant											
	TT	108 (50.2)	196 (41.2)	1.00	-	0.052	1.00	-	**0.09**	72.00	70.40-73.60	-
	TC	91 (42.3)	226 (47.5)	0.73	0.52-1.02		0.81	0.52-1.25		72.00	69.60-74.40	0.58
	CC	16 (7.4)	54 (11.3)	**0.54**	**0.29-0.99**		**0.44**	**0.20-0.95**		74.00	67.60-80.40	0.68
	Dominant											
	TT	108 (50.2)	196 (41.2)	1.00	-	**0.027**	1.00	-	0.13	72.00	70.40-73.60	-
	TC-CC	107 (49.8)	280 (58.8)	**0.69**	**0.50-0.96**		0.73	0.48-1.10		72.00	70.18-73.82	0.70
	Recessive											
	TT-TC	199 (92.6)	422 (88.7)	1.00	-	0.11	1.00	-	**0.049**	72.00	70.58-73.42	-
	CC	16 (7.4)	54 (11.3)	0.63	0.35-1.13		**0.49**	**0.23-1.03**		74.00	67.60-80.40	0.58
	Overdominant											
	TT-CC	124 (57.7)	250 (52.5)	1.00	-	0.21	1.00	-	0.76	72.00	70.46-73.54	-
	TC	91 (42.3)	226 (47.5)	0.81	0.59-1.12		0.94	0.62-1.42		72.00	69.60-74.40	0.48
	Log-additive	-		**0.73**	**0.57-0.94**	**0.015**	**0.72**	**0.52-0.99**	**0.041**	-		
rs1751031	Codominant											
	AA	154 (69.4)	296 (62.3)	1.00	-	0.10	1.00	-	0.073	72.00	70.25-73.75	-
	AG	59 (26.6)	164 (34.5)	**0.69**	**0.48**		**0.61**	**0.39-0.95**		72.00	69.72-74.28	0.66
	GG	9 (4.0)	15 (3.2)	1.15	0.49-2.70		0.57	0.17-1.92		78.00	69.81-86.19	0.29
	Dominant											
	AA	154 (69.4)	296 (62.3)	1.00	-	0.068	1.00	-	**0.022**	72.00	70.25-73.75	**-**
	AG-GG	68 (30.6)	179 (37.7)	0.73	0.52-1.03		**0.60**	**0.39-0.94**		72.00	70.08-73.93	0.46
	Recessive											
	AA-AG	213 (96.0)	460 (96.8)	1.00	-	0.55	1.00	-	0.52	72.00	70.61-73.39	-
	GG	9 (4.0)	15 (3.2)	1.30	0.56-3.01		0.68	0.21-2.24		78.00	69.81-86.19	0.31
	Overdominant											
	AA-GG	163 (73.4)	311 (65.5)	1.00	-	**0.034**	1.00	-	**0.036**	72.00	70.28-73.72	**-**
	AG	59 (26.6)	164 (34.5)	**0.69**	**0.48-0.98**		**0.62**	**0.40-0.98**		72.00	69.72-74.28	0.79
	Log-additive	-		0.81	0.61-1.09	0.17	**0.65**	**0.44-0.96**	**0.028**	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: Confidence Interval. Values in **bold** are statistically significant ($P<0.05$).

Table 5 (cont.). Genotype frequencies among gastric cancer cases and controls, risk estimates for the involvement of *PTGS2, ABCC4, HPGD, and SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype frequencies		Univariate analysis			Multivariate analysis			Age at diagnosis		
		Cases, *n* (%)	Controls, *n* (%)	OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
HPGD												
rs2303520	Codominant											
	GG	143 (64.4)	339 (71.4)	1.00	-	**0.037**	1.00	-	0.065	72.00	70.89-73.11	-
	GA	76 (34.2)	122 (25.7)	**1.48**	**1.04-2.09**		**1.61**	**1.02-2.54**		72.00	69.12-74.88	0.83
	AA	3 (1.4)	14 (3.0)	0.51	0.14-1.79		0.51	0.11-2.34		69.00	64.20-73.80	0.61
	Dominant											
	GG	143 (64.4)	339 (71.4)	1.00	-	0.066	1.00	-	0.086	72.00	70.89-73.11	-
	GA-AA	79 (35.6)	136 (28.6)	1.38	0.98-1.93		1.47	0.95-2.29		72.00	69.05-74.96	0.92
	Recessive											
	GG-GA	219 (98.7)	461 (97.0)	1.00	-	0.18	1.00	-	0.26	72.00	70.63-73.37	-
	AA	3 (1.4)	14 (3.0)	0.45	0.13-1.59		0.45	0.10-2.04		69.00	64.20-73.80	0.61
	Overdominant											
	GG-AA	146 (65.8)	353 (74.3)	1.00	-	**0.021**	1.00	-	**0.031**	72.00	70.87-73.13	-
	GA	76 (34.2)	122 (25.7)	**1.51**	**1.07-2.13**		**1.65**	**1.05-2.59**		72.00	69.12-74.88	0.81
	Log-additive	-		1.21	0.90-1.64	0.21	1.26	0.86-1.84	0.24	-		
SLCO2A1												
rs10935090	Codominant											
	CC	162 (73.0)	378 (79.6)	1.00	-	0.13	1.00	-	**0.026**	73.00	71.81-74.19	-
	CT	54 (24.3)	90 (18.9)	1.40	0.95-2.06	0.13	1.46	0.90-2.39	**0.026**	**70.00**	**67.56-72.44**	**0.034**
	TT	6 (2.7)	7 (1.5)	2.00	0.66-6.04		**4.68**	**1.32-16.61**		**62.00**	**59.61-64.39**	**<0.001**
	Dominant											
	CC	162 (73.0)	378 (79.6)	1.00	-	0.054	1.00	-	**0.038**	73.00	71.81-74.19	-
	CT-TT	60 (27.0)	97 (20.4)	1.44	1.00-2.09		**1.65**	**1.03-2.63**		**70.00**	**67.93-72.07**	**0.007**
	Recessive											
	CC-CT	216 (97.3)	468 (98.5)	1.00	-	0.28	1.00	-	**0.026**	72.00	70.87-73.14	-
	TT	6 (2.7)	7 (1.5)	1.86	0.62-5.59		**4.30**	**1.22-15.16**		**62.00**	**59.61-64.39**	**<0.001**
	Overdominant											
	CC-TT	168 (75.7)	385 (81.0)	1.00	-	0.11	1.00	-	0.19	73.00	71.807-74.193	-
	CT	54 (24.3)	90 (18.9)	1.37	0.94-2.02		1.39	0.86-2.27		70.00	67.56-72.44	0.057
	Log-additive	-		**1.40**	**1.01-1.95**	**0.044**	**1.69**	**1.12-2.53**	**0.012**	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: Confidence Interval. Values in **bold** are statistically significant (P<0.05).

Table 5 (cont.). Genotype frequencies among gastric cancer cases and controls, risk estimates for the involvement of *PTGS2, ABCC4, HPGD, and SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype frequencies		Univariate analysis			Multivariate analysis			Age at diagnosis		
		Cases, *n* (%)	Controls, *n* (%)	OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs11915399	Codominant											
	CC	159 (71.6)	326 (68.6)	1.00	-	0.72	1.00	-	0.12	72.00	70.27-73.73	-
	CT	57 (25.7)	135 (28.4)	0.87	0.60-1.24		**0.61**	**0.38-0.99**		73.00	71.47-74.53	0.13
	TT	6 (2.7)	14 (3.0)	0.88	0.33-2.33		0.75	0.22-2.63		74.00	59.93-88.07	0.52
	Dominant											
	CC	159 (71.6)	326 (68.6)	1.00	-	0.42	1.00	-	**0.043**	72.00	70.27-73.73	-
	CT-TT	63 (28.4)	149 (31.4)	0.87	0.61-1.23		**0.62**	**0.39-0.99**		73.00	71.53-74.47	0.11
	Recessive											
	CC-CT	216 (97.3)	461 (97.0)	1.00	-	0.86	1.00	-	0.81	72.00	70.64-73.37	-
	TT	6 (2.7)	14 (3.0)	0.91	0.35-2.41		0.86	0.25-2.96		74.00	59.93-88.07	0.59
	Overdominant											
	CC-TT	165 (74.3)	340 (71.6)	1.00	-	0.45	1.00	-	**0.045**	72.00	70.19-73.81	-
	CT	57 (25.7)	135 (28.4)	0.87	0.61-1.25		**0.62**	**0.38-1.00**		73.00	71.47-74.53	0.16
	Log-additive	-		0.89	0.65-1.21	0.45	0.69	0.46-1.03	0.065	-		
rs9821091	Codominant											
	GG	87 (39.2)	179 (37.7)	1.00	-	0.32	1.00	-	**0.045**	72.00	69.77-74.23	-
	GA	97 (43.7)	232 (48.8)	0.86	0.61-1.22		**0.81**	**0.52-1.28**		73.00	71.43-74.57	0.11
	AA	38 (17.1)	64 (13.5)	1.22	0.76-1.97		**1.75**	**0.95-3.20**		71.00	68.17-73.83	0.16
	Dominant											
	GG	87 (39.2)	179 (37.7)	1.00	-	0.70	1.00	-	0.96	72.00	69.77-74.23	-
	GA-AA	135 (60.8)	296 (62.3)	0.94	0.68-1.30		0.99	0.65-1.50		72.00	70.56-73.44	0.42
	Recessive											
	GG-GA	184 (82.9)	411 (86.5)	1.00	-	0.21	1.00	-	**0.02**	72.00	70.76-73.24	-
	AA	38 (17.1)	64 (16.5)	1.33	0.86-1.12		**1.95**	**1.12-3.40**		**71.00**	**68.17-73.83**	**0.017**
	Overdominant											
	GG-AA	125 (56.3)	243 (51.2)	1.00	-	0.20	1.00	-	0.085	71.00	69.06-72.94	-
	GA	97 (43.7)	232 (48.8)	0.81	0.59-1.12		0.70	0.46-1.05		**73.00**	**71.43-74.57**	**0.018**
	Log-additive	-		1.05	0.83-1.32	0.70	1.19	0.89-1.61	0.24	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: Confidence Interval. Values in **bold** are statistically significant (P<0.05).

4.2.2. Functional characterization of the GC risk-associated biomarkers

We then questioned if the genetic variants associated with GC susceptibility could be determinants of mRNA expression. We observed associations between two polymorphisms, the rs2303520 in *HPGD* gene and the rs11915399 in *SLCO2A1* gene, and the expression of the correspondent genes. As can be observed in **Figure 9**, the GA genotype is associated with a decrease in *HPGD* mRNA expression by a mean factor of 0.67 compared to the heterozygous genotype (4.40±0.16 vs 3.82±0.21, P=0.027) in "normal"-appearing mucosa samples.

Regarding the rs11915399 tagSNP in the *SLCO2A1* gene, represented in **Figure 10**, we found an increase in mRNA expression in the homozygous individuals for the minor T allele compared to both the carriers of the major C allele (2.07±0.83 vs 1.36±0.09, P=0.007) and the CC genotype alone (1.32±0.09, P=0.006) by a mean factor of 1.67 and 1.63, respectively.

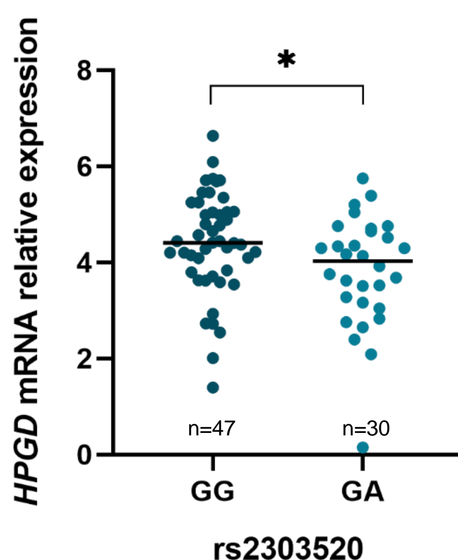

Figure 9. *HPGD* mRNA relative expression considering the genotypes of the rs2303520 G>A polymorphism. In "normal"-appearing mucosa, the GA genotype is associated with *HPGD* mRNA downregulation by a mean factor of 0.67. Lines represent median values of expression. *P<0.05.

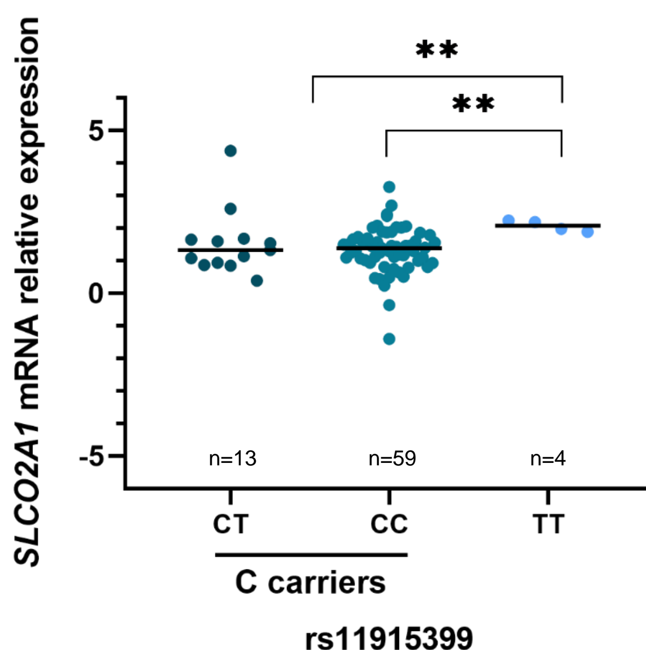

Figure 10. ***SLCO2A1*** **mRNA relative expression considering the genotypes of the rs11915399 C>T polymorphism.** In "normal"-appearing mucosa, the TT genotype is associated with *SLCO2A1* mRNA upregulation compared to the CC genotype and the carriers of the C allele by a mean factor of 1.63 and 1.67, respectively. Lines represent median values of expression. **P<0.01.

4.2.3. Haplotype analysis

Since multiple SNPs were addressed within *ABCC4* and *SLCO2A1* genes, a haplotype analysis was performed, and the derived haplotypes frequencies are displayed in **Table 6.** Seven common haplotypes were described for *ABCC4* gene. The most frequent one (TTA) was present in 42% of controls and was used as the reference haplotype. Only the block containing the rs1678374C, rs1678405C, and rs1751031G alleles, CCG, showed an influence in GC susceptibility, presenting a 53% protection for GC onset, which is consistent with the individual SNP analysis (aOR=0.47; 95% CI: 0.23-0.93; P=0.032).

The reference haplotype for the *SLCO2A1* gene, CCG, was present in over 45% of controls. A 2.8-fold increased risk was observed for individuals carrying the block TCA (95% CI: 1.41-5.48; P=0.0034), which contains the alleles associated with increased risk in the individual analysis of the rs10935090 and rs9821091 genetic polymorphisms (minor T allele and minor A allele, respectively). The rs11915399C allele is also included in that block, which is coherent with the association reported between its opposing rs11925399T allele and GC protection. Summing up, for both genes, only the blocks containing the

alleles associated with GC susceptibility from all the addressed SNPs presented some type of significant association with this type of cancer.

Table 6. Haplotype frequencies between patients and controls and risk estimates for their involvement in gastric cancer onset.

Gene/Haplotype	% Cases	% Controls	aOR	95% CI	P value
ABCC4 [£]					
T-T-A	49.09	42.16	1.00	Reference	-
C-C-A	13.84	16.87	0.67	0.42-1.09	0.11
C-T-A	13.12	12.46	0.94	0.52-1.69	0.83
C-C-G	7.07	9.68	**0.47**	**0.23-0.93**	**0.032**
T-T-G	7.42	7.81	0.52	0.26-1.06	0.074
T-C-A	6.61	8.09	0.70	0.35-1.43	0.33
C-T-G	1.33	2.49	0.56	0.11-2.74	0.47
SLCO2A1 [¥]					
C-C-G	45.37	45.31	1.00	Reference	-
C-C-A	26.04	27.74	0.89	0.60-1.32	0.57
C-T-G	9.23	11.12	0.58	0.32-1.07	0.084
T-C-A	6.61	4.98	**2.78**	**1.41-5.48**	**0.0034**
T-C-G	6.44	0.48	0.91	0.44-1.87	0.80

aOR: odds ration adjusted for age; CI: confidence interval.
[£]SNPs order: rs1678374-rs11678405-rs1751031
[¥]SNPs order: rs10935090-rs11915399-rs9821091

4.2.4. Gene-“environment” interaction analysis

An MDR approach was carried out to assess all possible interactions between all the analyzed SNPs and only between the most meaningful SNPs, i.e., the polymorphisms associated with GC onset in the main analysis (rs689466 in *PTGS2* gene, rs1678374, rs1678405, rs1751031 in *ABCC4* gene, rs2303520 in *HPGD* gene, and rs10935090, rs1191599, and rs9821091 in *SLCO2A1* gene) and the best interactive models are summarized in **Table 7**. We further included in both analysis the age and sex variables. All the addressed models were significant at P<0.0001 and the highest CVC was observed for the model with one single factor in both cases (10/10). Nevertheless, regarding the models including all SNPs, the three-locus model presented the highest testing accuracy for predicting the development of GC (84.6%), despite presenting a lower CVC (6/10). That model included the polymorphisms rs5275 in *PTGS2* gene, rs9820625 in *SLCO2A1* gene, and age and was associated with a 30-fold increased GC risk.

Considering the best models including only the risk-associated SNPs, the three-factor interaction model also presented the highest testing accuracy of 80.7% with a CVC of 8/10 and included age and the SNPs rs689466 in *PTGS2* gene and rs1678374 in

ABCC4 gene. This gene-“environment” interaction was associated with a 17.6-fold increase in GC risk.

Table 7. MDR analysis for gastric cancer risk prediction, considering all tested SNPs and only SNPs associated with gastric cancer risk.

	CV accuracy	CV consistency	aOR	95% CI	P value
Best model – all SNPs					
rs689466	0.621	10/10	2.743	1.967-3.826	<0.0001
age, rs3742106	0.716	8/10	6.370	4.412-9.198	<0.0001
age, rs5275, rs9820625	0.846	6/10	30.413	19.168-48.256	<0.0001
Best model – risk-associated SNPs					
rs689466	0.621	10/10	2.743	1.967-3.826	<0.0001
age, rs1678374	0.687	5/10	4.953	3.434-7.143	<0.0001
age, rs689466, rs1678374	0.807	8/10	17.581	11.672-26.482	<0.0001

MDR: multifactor dimensionality reduction, CV: cross-validation, aOR: odds ratio adjusted for age; CI: confidence interval.

Upon performing the FDR analysis to address multiple testing, none of the genetic biomarkers previously associated with GC susceptibility retained its statistical significance.

4.3. Assessment of the *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* mRNA expression in GC (Study 2)

To further clarify the role of the PGE_2 pathway in GC, we sought to investigate the mRNA expression of the genes encoding COX-2, MRP4, 15-PGDH, and PGT in “normal”-appearing and tumoral mucosa of GC patients from a Caucasian population. The RNA was successfully extracted from a total of 94 “normal”-appearing mucosa samples and 89 tumorous mucosa samples. Overall, *PTGS2* gene was found to be overexpressed in tumor samples compared to normal mucosa (-0.63±0.16 vs -1.95±0.15; P<0.0001), leading to a 2.51-fold increase in mRNA expression, as can be observed in **Figure 11**. On the other hand, the *HPGD* and *SLCO2A1* genes were found to be downregulated in GC mucosa by a mean factor of 0.10 and 0.37, respectively (0.97±0.19 and -0.02±0.17 vs 4.27±0.12 and 1.40±0.08 in normal samples, respectively; P<0.0001 for both genes). We found no statistically significant difference in *ABCC4* mRNA expression (1.59±0.79 in normal vs 1.52±0.12 in tumoral samples; P=0.822).

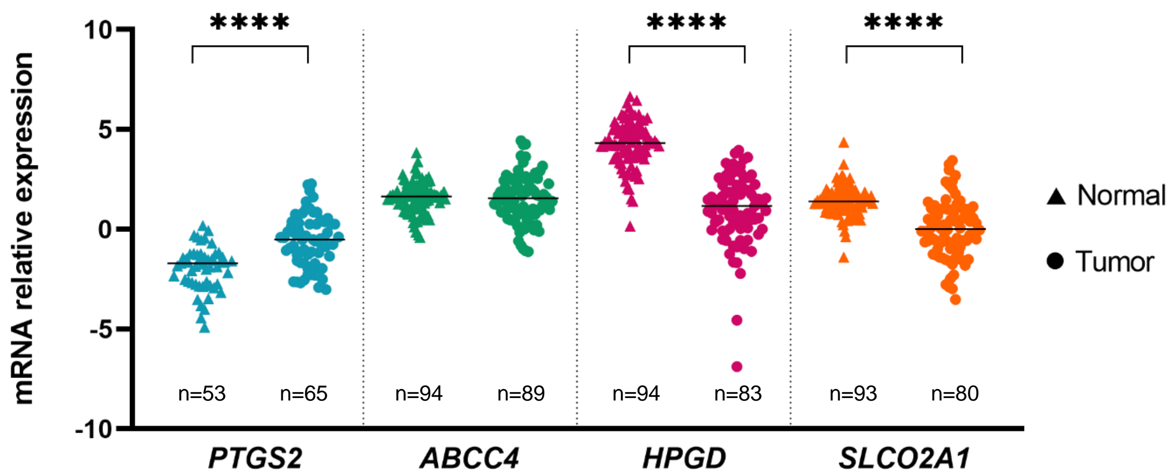

Figure 11. mRNA expression of the PGE_2 pathway in GC. *PTGS2* is upregulated in tumor samples comparing to normal samples by a mean factor of 2.51, whereas *HPGD* and *SLCO2A1* are downregulated in tumorous mucosa by a mean factor of 0.10 and 0.37, respectively. Lines represent median values of expression. ****P<0.0001.

The expression of these four genes did not differ across early (I and II) and advanced (III and IV) stages of the disease, as displayed in **Figure 12**.

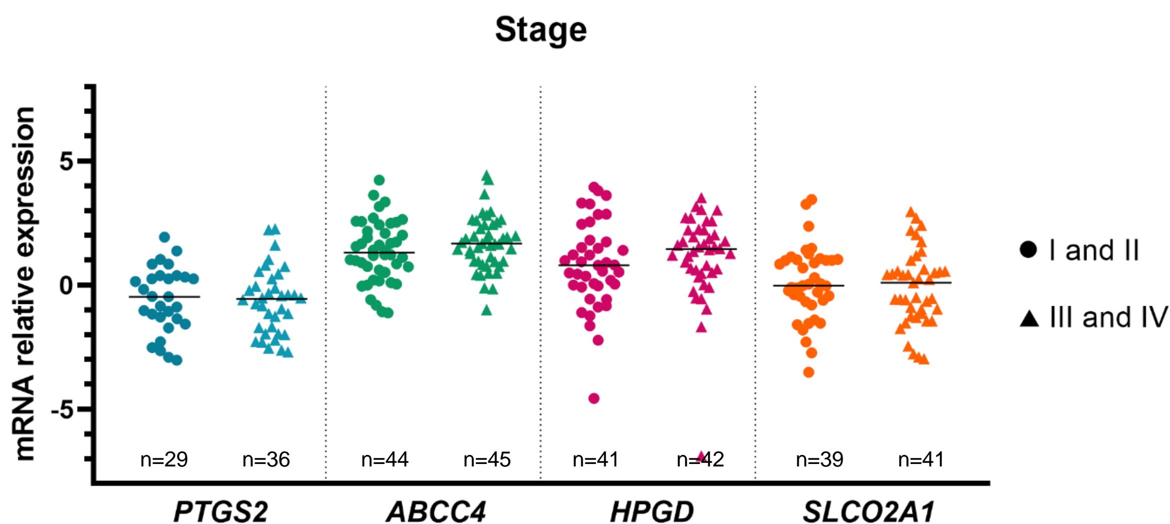

Figure 12. mRNA expression across GC stages. No statistically significant differences are found in the mRNA expression of the *PTGS2, ABCC4, HPGD,* and *SLCO2A1* genes across GC stages. Lines represent median values of expression.

We then evaluated the expression profile of the PGE_2 pathway across different regions of the stomach. In cardia and GEJ, we found a downregulation of the *HPGD* and *SLCO2A1* genes in tumorous mucosa (1.19±0.58 vs 3.74±0.63 in normal mucosa, P=0.0005 for *HPGD* gene; 0.37±0.70 vs 0.63±0.51 in normal mucosa, P=0.0007 for *SLCO2A1* gene), as displayed in **Figure 13**. The fundus and corpus and the antrum and transition corpus-antrum demonstrated similar patterns (**Figure 14 and Figure 15**). We found a 2.68-fold increased COX-2 mRNA in tumoral mucosa in the fundus and corpus, and a 2.40-fold increase in the antrum and transition (-0.47±0.31 vs -1.90±0.19 in normal samples of the fundus and corpus, P=0.0024; -0.55±0.21 vs -1.82±0.18 in normal samples of the antrum and corpus-antrum transition, P<0.0001). Consistent with the overall analysis, a downregulation of *HPGD* and *SLCO2A1* was observed in GC located at the fundus and corpus and antrum and the corpus-antrum transition (0.62±0.57 vs 4.78±0.31 in normal samples in the fundus and corpus, P<0.0001; 0.93±0.23 vs 4.25±0.14 in the antrum and transition corpus-antrum, P<0.0001, for the *HPGD* gene and 0.19±0.38 vs 1.16±0.16 in normal-appearing mucosa of the fundus and corpus, P=0.0021; -0.12±0.22 vs 1.36±0.08 in the antrum and transition corpus-antrum, P<0.0001 for the *SLCO2A1* gene). Concerning incisura angularis, we only had mRNA expression data from three samples in each histological type and no statistically significant differences were found (data not shown).

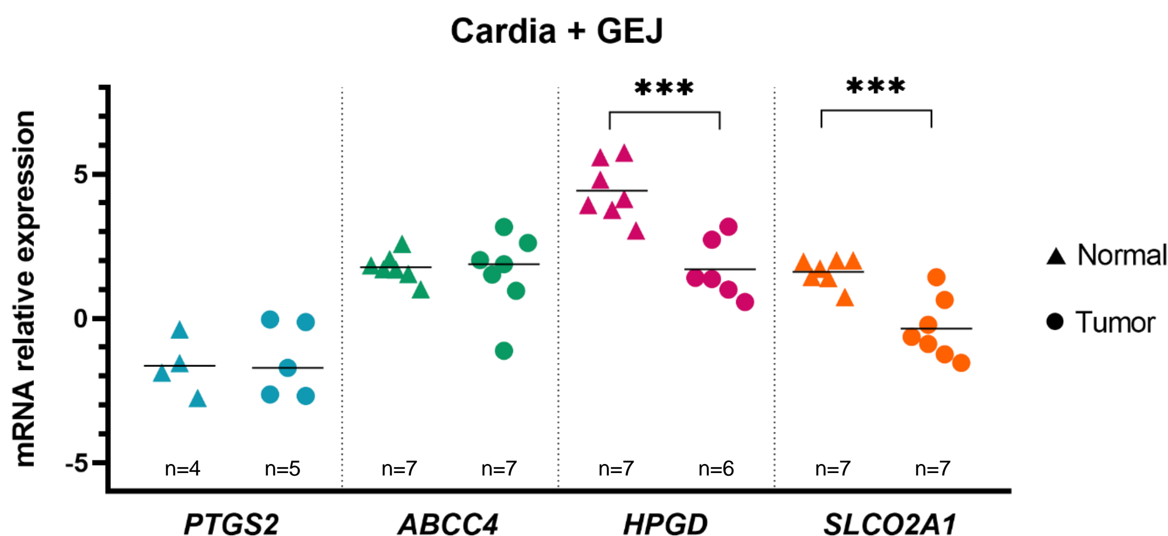

Figure 13. mRNA expression according to localization: cardia and GEJ. *HPGD* and *SLCO2A1* genes are downregulated in tumor samples compared to “normal”-appearing mucosa samples by a mean-factor of 0.15 and 0.28, respectively. Lines represent median values of expression. ***P<0.001.

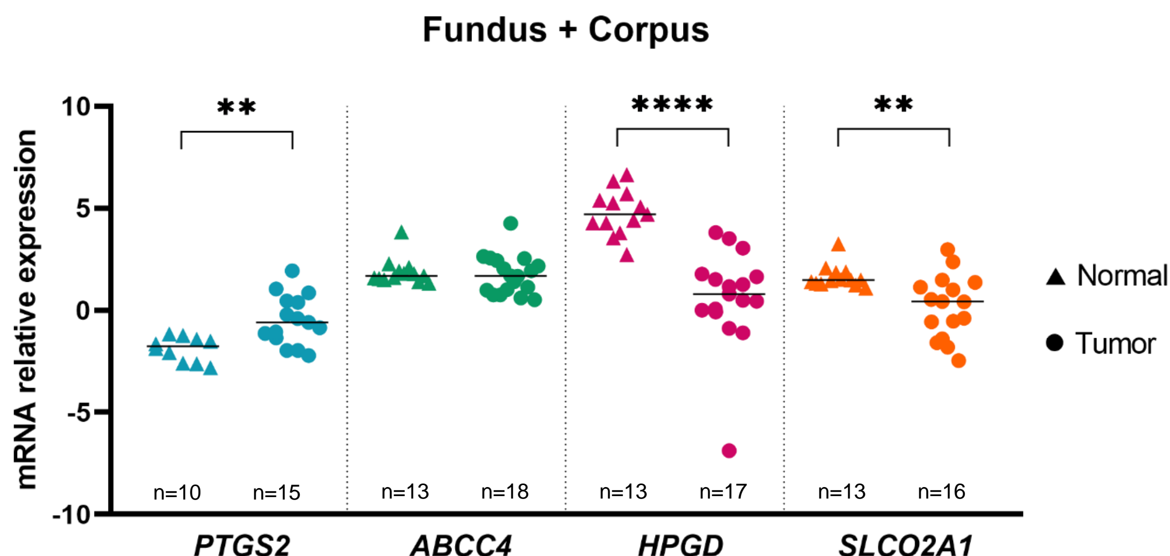

Figure 14. mRNA expression according to localization: fundus and corpus. *PTGS2* is upregulated in tumor samples by a mean-factor of 2.68, whereas *HPGD* and *SLCO2A1* genes are downregulated compared to "normal"-appearing mucosa samples by a mean-factor of 0.14 and 0.36, respectively. Lines represent median values of expression. **P<0.01 ****P<0.0001.

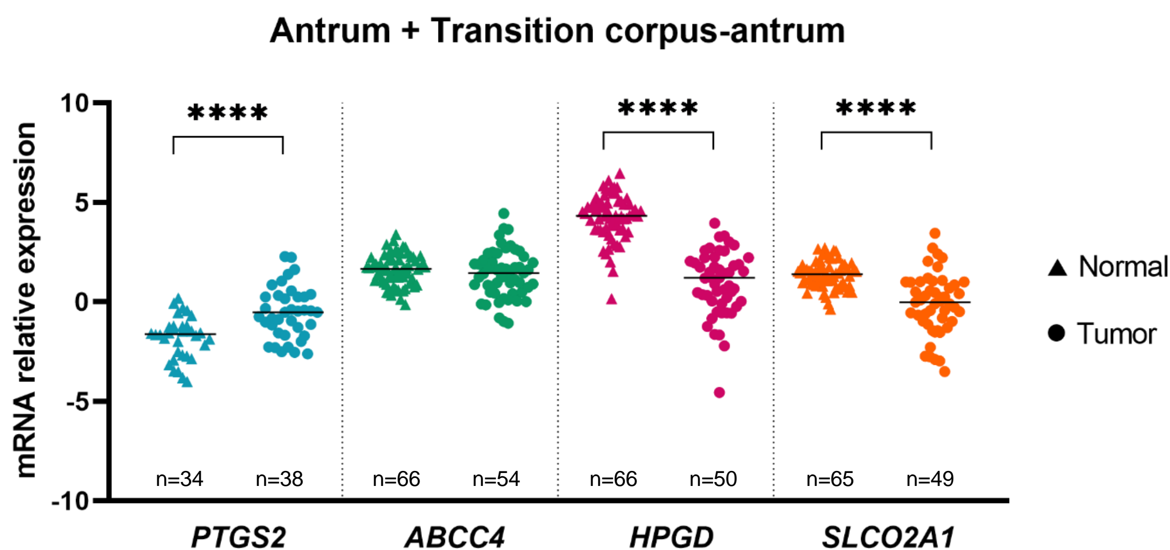

Figure 15. mRNA expression according to localization: antrum and transition corpus-antrum. *PTGS2* is upregulated in tumor samples by a mean-factor of 2.40, whereas *HPGD* and *SLCO2A1* genes are downregulated compared to "normal"-appearing mucosa samples by a mean-factor of 0.10 and 0.36, respectively. Lines represent median values of expression. ****P<0.0001.

A similar expression pattern was observed among males (**Figure 16**) and females (**Figure 17**), although more noticeably in the latter group. Interestingly, the females presented a statistically significant decrease in *ABCC4* mRNA expression in tumor samples (1.13±0.17 vs 1.63±0.12 in normal mucosa, P=0.025) by a mean factor of 0.71, which was not observed in males. In fact, when we compared the mRNA expression of these proteins between females and males in tumorous mucosa, as displayed in **Figure**

18, we found an *ABCC4* downregulation in females (1.13±0.17 vs 1.85±1.17 in males, P=0.038) and a *PTGS2* upregulation (-0.28±0.22 vs -0.99±0.23 in males, P=0.028).

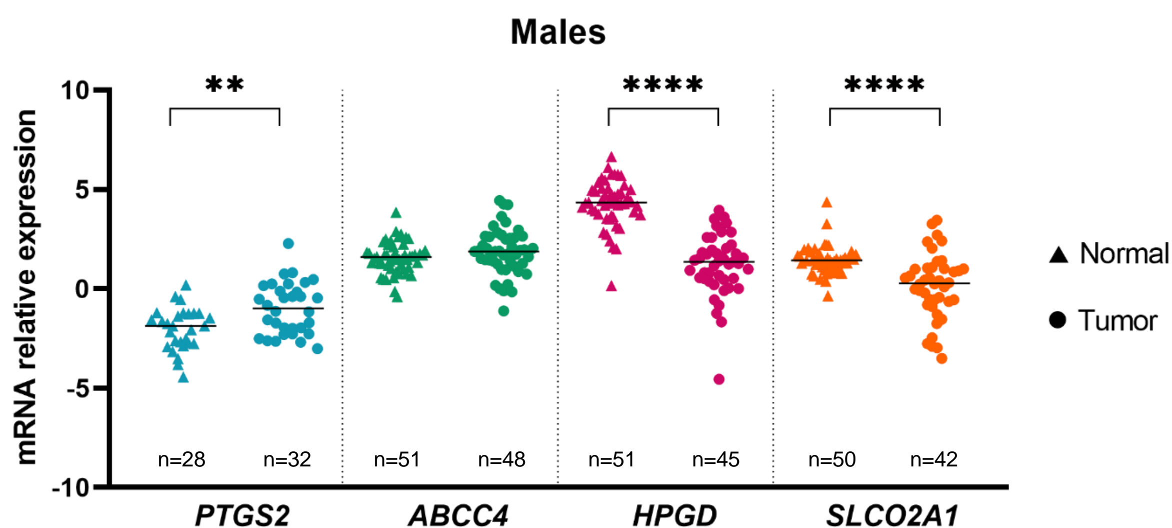

Figure 16. mRNA expression according to gender: males. *PTGS2* is upregulated in tumor samples by a mean-factor of 2.05, whereas *HPGD* and *SLCO2A1* genes are downregulated compared to "normal"-appearing mucosa samples by a mean-factor of 0.12 and 0.41, respectively. Lines represent median values of expression. **P<0.01; ****P<0.0001.

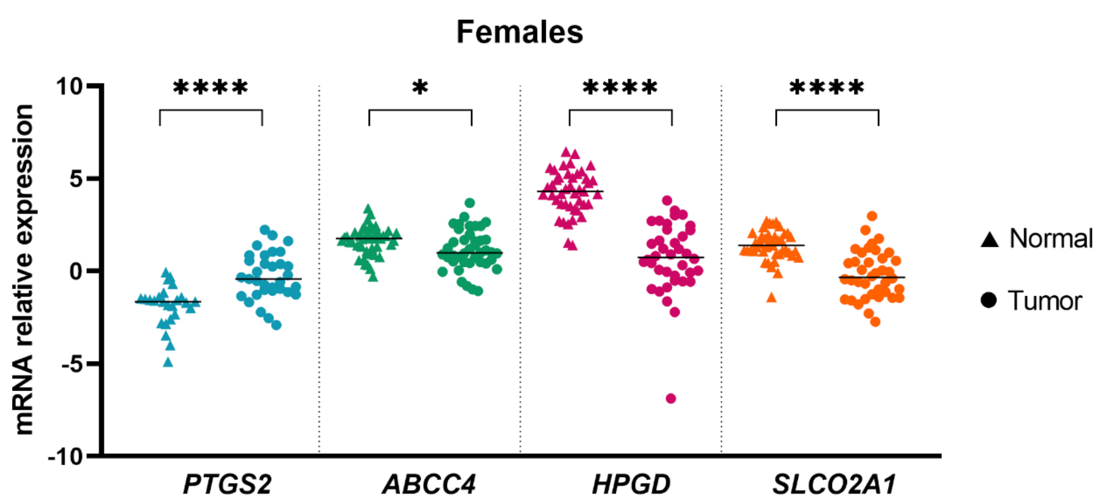

Figure 17. mRNA expression according to gender: females. *PTGS2* is upregulated in tumor samples by a mean-factor of 2.64, whereas *ABCC4, HPGD,* and *SLCO2A1* genes are downregulated compared to "normal"-appearing mucosa samples by a mean-factor of 0.71, 0.08 and 0.34, respectively. Lines represent median values of expression. *P< 0.05; ****P<0.0001.

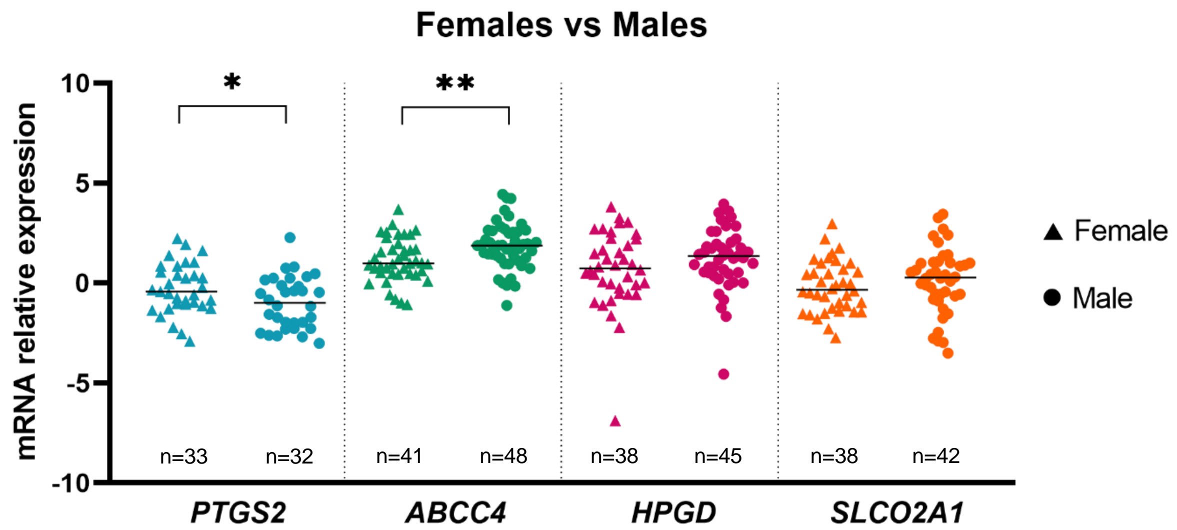

Figure 18. mRNA expression in tumoral mucosa according to gender. In females, *PTGS2* is upregulated by a mean-factor of 1.64, whereas *ABCC4* is downexpressed in the tumoral cells by a mean factor of 0.61 compared to males. Lines represent median values of expression. *P<0.05; **P<0.01.

5. DISCUSSION

In the 21st century, cancer is expected to rank as the leading cause of death and the main responsible for hampering the increase in life expectancy worldwide [1]. Regarding GC, countries with the highest incident rates also present the highest proportions in relation to all diagnosed cancers [4]. While Portugal as a whole presents low to intermediate incidence, although it is the highest among Western European countries, the Northern region of our country has the third-highest proportion of GC in relation to other types of cancer, following Japan and Ukraine, presenting about two times the expected values for Spain [4, 165, 166]. The relatively lower incidence and mortality rates in Western Europe and North America make the populational screening observed in Asia unjustifiable and, despite the cost-effectiveness of *H. pylori* eradication in high-risk areas, its resistance and adverse effects are still a concern [19, 42]. However, the surveillance of high-risk patients has been shown to be cost-effective in this population [12, 167]. Thus, in Western countries such as Portugal, the most effective approach to reduce GC mortality is presumably through the identification of risk factors that allow personalized screening and surveillance [168].

The pleiotropic activities of PGE_2/COX-2 pathway and their effect on cancer progression have been reviewed and explored throughout the years [51, 59, 169, 170]. This pathway involves PGE_2 synthesis via COX-2 within the cell and its transport to the extracellular milieu by MRP4, where it is able to interact with PG receptors (EP1-4) and exert its effects [59, 171]. Inversely, PGT is responsible for the transport of this PG back into the cell so it can be catabolized and inactivated by 15-PGDH [51]. Dysregulation of this pathway due to COX-2/MRP4 overexpression and PGT/15-PGDH downregulation has been shown to lead to the accumulation of PGE_2 in the extracellular microenvironment and, therefore, to contribute to its nefarious effects [51, 52]. Inhibitors of the COX enzymes, such as the aspirin, are potential agents for chemoprevention of this type of cancer, but the association of this NSAID with excess bleeding or gastrointestinal damage remains a concern [172].

Considering that the genetic and molecular signatures differ across ethnic populations and the fact that most published studies focus on Asian GC patients, the role of PGE_2 pathway remains unclear in Caucasian patients. Our group had already explored the involvement of genetic variants in this pathway in colorectal cancer development [128]. In this study, we hypothesized that the PGE_2 pathway, namely the COX-2, MRP4, 15-PGDH, and PGT proteins, is implicated in GC development in Caucasians. For this purpose, a hospital-based case-control study was implemented in the major Oncology Institute of the Northern region of Portugal. Due to the low recruitment rate, we had to resort to FFPE samples archived at the Pathology department at IPO-Porto to characterize our group of GC patients.

The estimated number of biological samples archived worldwide surpasses one billion and most of them are FFPE tissues, which have been well preserved, allowing to deepen the knowledge of complex diseases, such as cancer [173]. FFPE blocks can be stored for tens of years and allow extraction of both RNA and DNA, although nucleic acids degrade in a time-dependent manner due to the decrease in pH [174]. However, molecular analysis with this type of tissue remains difficult due to the method of preservation and to the lack of standardized guidelines [173].

Nowadays, PCR techniques allow the use of FFPE tissue but they require optimization, short amplicons and rely on the quantity and quality of the extracted RNA [175, 176]. The latter is determined by the fixation process, including handling and time of fixation, and depends on RNA fragmentation, cross-linking with peptide fragments and base modification, which we could not control [176]. The quantity depends on the extraction method and optimization of the real-time PCR [176]. It is known that the downstream applications of FFPE-derived RNA require a higher amount of starting material compared to other types of tissue, such as frozen tissue, because formalin fixation results in a reduction of approximately 70% in the quantity of nucleic acid obtained and in the predominant damage of the ends of the RNA molecules [175-177]. Nevertheless, those quantities are obtainable from FFPE samples and RNA can be released from the cross-linked proteins and nucleic acids by proteinase K digestion steps combined with heating, which break the formalin-formed methylene bridges [139]. Walter *et al.* [178] even described successful and reproducible RT and PCR amplification for FFPE samples, showing no inhibitory effect of the formalin. Regarding genotype characterization, previous studies have reported concordance rates superior to 93% between germline DNA and FFPE samples using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with Sequenom iPLEX technology [179].

Regarding our group of GC patients, we observed an enrichment in stage II and III tumors relatively to stages I and IV. That might be explained by the fact that, many times, early and localized lesions of the stomach can be treated by endoscopic mucosal resection (mucosectomy), without resorting to surgery [180]. On the other hand, more advanced cancers may involve distant metastatic lesions, usually incurable, and/or patients more debilitated, so surgery might not be an effective procedure for those cases, resulting in a reduction of FFPE samples of that stage.

Even though we did not match controls to cases, the effect of potential confounding variables in the statistical analysis, such as age, was minimized through multivariate analysis.

Considering these methodological considerations, we will proceed with an overview of the main findings of this study.

For the genomic characterization of the PGE_2 pathway we applied a tagSNP approach, which allowed us to capture the majority of SNP variation in a genome region, reducing the necessity of a large amount of sample and the genotyping costs [181]. The tagSNPs transferability in distinct populations has been explored in several studies [126, 182-184]. Our panel was retrieved from the CEU population, represented by Utah residents with Northern and Western Europe ancestry, of the International HapMap project [185].

In total, we found eight polymorphisms involved in GC susceptibility: one in *PTGS2* gene, three in *ABCC4* gene, one in *HPGD* gene, and three in *SLCO2A1* gene. Regarding *PTGS2*, rs689466A>G, we found an association between the minor G allele and 3-fold increased risk for GC onset. It is known that this SNP is located in the promoter region of the gene at -1195 bp from the transcription start site, which is rich in several *cis*-regulatory elements involved in *PTGS2* transcription [186]. No association was found between the rs689466 polymorphism and mRNA expression. However, we had a limited amount of mRNA expression results for that gene. Furthermore, the presence of the G allele has been previously associated with higher transcriptional activity of *PTGS2* in colorectal cell lines and the implication of this SNP in colorectal cancer susceptibility had already been reported by our group [187]. This result contradicts the reports of a meta-analysis by Luo *et al*. [188], which identifies the rs689466A allele as an increased risk genetic biomarker for GC. Additionally, the rs689466A allele has also been shown to create a binding-site for a transcription-factor, c-MYB, which enhances *PTGS2* transcriptional activity [189]. Also, another meta-analysis by Wang *et al*. [190] reports an association between COX-2 rs689466A allele and increased risk of several cancers, including hepatocellular carcinoma, pancreatic, and gastric cancer. Nevertheless, it should be noted that the studies included in these meta-analyses were performed almost exclusively in Asian populations. Genetic polymorphisms in regulatory regions are expected to be modulated by the bioavailability of nuclear proteins and activated pathways, which are suggested to be distinct, at least, across ethnicities and disease models.

Considering the other *PTGS2* gene polymorphisms, we only tested the rs5275 SNP, located in the 3' untranslated region (3'-UTR), and no association was observed in our population. However, an involvement of this genetic variation in GC in high-risk populations, such as the Chinese, and in breast cancer in a Caucasian population has already been reported [191-193]. The rs20417 polymorphism was excluded from our analysis due to genotyping failure, although it has been suggested as a susceptibility biomarker for GC in Caucasian patients with gastric atrophy or intestinal metaplasia [194]. Furthermore, whereas some studies showed no association between this SNP and COX-

2 expression, others reported that the rs20417GG genotype was associated with a higher COX-2 expression *in vitro* and in colorectal patients [195-197].

In the *ABCC4* gene, the rs1678374, rs1678405, and rs1751031 polymorphisms were found to be associated with GC susceptibility in this study. Concerning rs1678374T>C, we observed an association between the CC genotype and a 50% risk reduction for GC development. There are no reports suggesting an influence of this SNP in genetic susceptibility for a disease, but it is known to be located within an intron (intron 9) [198]. Even though this type of mutation does not affect the sequence of amino acids of a protein, genetic variation in noncoding DNA sequences, such as introns, can have important functional and regulatory roles [199, 200]. On the other hand, associations between rs1678405C allele and AG genotype of the rs1751031 SNP with colorectal cancer protection were previously reported by our group, which is consistent with our results [128, 131]. These two polymorphisms are also located in introns, as well as all the SNPs belonging to their blocks.

Comparably to rs1678374 *ABCC4* polymorphism, there are no studies reporting an association between rs2303520G>A SNP in *HPGD* gene and disease development. Nevertheless, according to our results, carriers of the GA genotype presented an increased risk for GC. In fact, we could also observe an association between individuals carrying this genotype and a decrease in *HPGD* mRNA expression, which may impair PGE_2 inactivation within the cell. Therefore, this may contribute to an increase in its concentration and, consequently, to its nefarious effects in the extracellular milieu, thus supporting the observed increased risk for GC. We investigated the functional role of the actualized members of the same block (the blocks presented in Table S1 had been searched a few years ago) and found that the rs9312555 polymorphism is located in a 3'-UTR and, thus, might be implicated in the modification of protein and microRNA binding sites in regulatory elements and in a panoply of functional consequences [201]. We found no association between the rs8752 SNP and cancer susceptibility, although it has been linked to colorectal, prostate, and breast cancer risk [133-135]. The rs2555639 polymorphism in *HPGD* gene failed to be converted to the Sequenom platform, yet it has been associated with colon cancer susceptibility, with the rs2555639T allele being responsible for the downregulation of this protein [129].

Regarding the gene encoding the PGT protein, the *SLCO2A1*, we reported a four and two-fold increased risk for GC onset in our population for the rs10935090TT and the rs9821091AA genotypes, respectively. The former is known to be located in exon 1 and to represent a synonymous variant and the latter to be located in an intron [199]. On the other hand, carriers of the T allele for the rs11915399C>T polymorphism exhibited a decreased risk for GC. Corroborating these results, an association was found between

that genotype and an increase in *SLCO2A1* mRNA expression compared to carriers of the C allele and the CC genotype alone, contributing to PGE_2 transport back into the cell by PGT and, consequently, its inactivation and a decrease in GC risk.

In the epistasis analysis, we observed that the models with the highest CV accuracy included three factors, even though they did not present the best CVC (6/10 and 8/10). Overall, the best three-factor model revealed an 80.7% CV accuracy for the interaction between age, rs689466, and rs1678374 genetic polymorphisms and was associated with a 17.6-fold increased risk for GC development. This approach in complex diseases may help understand a likely source of heritability, allowing the definition of genetic and gene-environment signatures for the development of GC, which may represent an important key to GC prevention, and, with further studies, might provide important clues on the inter-regulation of these genes [202].

A major limitation inherent to our study is the multiple testing problem, which we corrected using FDR, but, due to our restricted statistical power, none of the SNPs we found to be associated with GC retained their statistical significance. To overcome that, we would need to increase the number of samples in future studies, so we can increase the statistical power and, thus, the precision of our results.

Next, we evaluated the mRNA expression of the four proteins involved in the PGE_2 pathway, COX-2, MRP4, 15-PGDH, and PGT. Our results corroborate the findings of most studies, reporting an increase in *PTGS2* mRNA expression in tumoral samples compared to the normal mucosa and, on the other hand, a downregulation of the *HPGD* and *SLCO2A1* genes, in a gender-independent manner. That pattern was similar in all the studied regions of the stomach, with the exception of the cardia and GEJ, where we did not find a statistically significant difference in the COX-2 mRNA expression between the two types of samples. Despite that, no differences were found in the mRNA levels of *ABCC4* gene. Interestingly, when we compared the mRNA values in the tumoral mucosa between males and females, we found a decrease in MRP4 mRNA expression in females in our population. We could not find an explanation for this distribution in the published literature. However, this contradicts the findings in the kidney and liver of female mice, which showed higher MRP4 levels [203]. In the former organ of those rodents, MRP4 appeared to be repressed by the male hormone 5α-dihydroxytestosterone and by male-pattern growth-hormone secretion [204]. Further studies are necessary to understand if there are other factors contributing to the difference in *ABCC4* mRNA expression we observed across males and females.

In the published literature, COX-2, undoubtedly the most studied protein of this pathway in a variety of cancers, is found upregulated in most tumors analyzed by a variety of methods (Northern blot, immunoblotting, and RT-PCR) [205]. In GC, the majority of

studies focusing on this protein expression and role involve Asian populations [206-214], but some studies involve North American [215], African [216], and European patients [90, 95, 217, 218].

The expression and role of 15-PGDH in GC are still somewhat controversial. While some studies report a decreased expression of this protein in gastric malignancies compared to normal gastric tissues, others show no such difference [47, 219-223]. It is noteworthy that all these studies were performed in either Chinese or Korean populations. Moreover, 15-PGDH has been regarded as a tumor suppressor by some reports and has been associated with the development of gastric carcinoma by inducing apoptosis and cell cycle arrest [47, 220, 221, 223]. The correlation between 15-PGDH expression and some pathological findings has also been controversial. Some authors defend no correlation between the expression of this enzyme and tumor-node-metastasis stage, vascular invasion, and tumor histologic type [220, 224], whereas a study by Seo *et al.* [222] reports significant correlation between 15-PGDH expression and T and N stage, pathologic type, metastasis, vascular, lymphatic, and perineural invasion, and palliative gastrectomy. In addition, that study associates the expression of 15-PGDH with the 5-year gastric-cancer-specific survival, but it does not classify it as an independent prognostic factor [222]. On the other hand, Tatsuwaki *et al.* [98] performed a multivariate analysis and concluded that reduction of 15-PGDH expression could, in fact, be an independent predictor of poor survival and it was correlated with differentiation, disease stage, and prognosis. *H. pylori* infection has also been related to this protein, as it appeared to promote gastric carcinogenesis by modulating both 15-PGDH and COX-2 mRNA expression and protein synthesis [209, 225].

As mentioned previously, not much is known about the PGE_2 transporters in GC. MRP4 role as a drug transporter, which may also contribute to cancer progression, has been explored in a variety of diseases. Moreover, it is known to transport PGE_2 out of the cell, increasing its extracellular levels, so an upregulation of this protein in tumorous samples would have been expected. Reduced PGT expression has been associated with increasing PGE_2 levels in the tumor microenvironment and, consequently, with tumor angiogenesis in GC [64]. Moreover, colocalization of this transporter and the PGE_2 receptor EP4 has been detected in the mucosa of both normal stomach and gastric carcinoma, suggesting a role of PGT in the PGE_2-mediated cellular effects [226]. In the same study, by Bujok *et al.* [226], a higher expression of the protein was found in GC tissue compared to normal tissue, but with no statistical significance. Takeda *et al.* [227] were the first to identify PGT expression as an independent predictor of poor prognosis in patients with GC. In that study, reduction of the transporter expression correlated with increased tumor angiogenesis and its suppression by specific siRNA promoted the

production of vascular endothelial growth factor (VEGF), a mediator of angiogenesis, induced by PGE_2 [227]. Furthermore, the immunohistochemical staining showed a diffuse PGT expression in normal gland epithelial cells of the stomach, similar to other expression patterns reported previously in normal intestinal cells and suggesting a strict regulation of PGE_2 concentration to maintain cellular homeostasis [227]. The authors presume that this homeostasis is impaired in gastric tumors due to the negative regulation of PGT and, consequently, the negative regulation of PGE_2 degradation, resulting in the enhancement of PGE_2 signaling and gastric tumorigenesis [227]. Contrary to those reports, a study by Nakanishi *et al.* [228] suggests an association between higher PGT expression in colorectal cancer and poor prognosis. The authors indicate a likely promotion of tumorigenesis by PGE_2 uptake into the endothelial cells via this transporter [228]. In ovarian cancer, both increased and reduced levels of PGT have been reported [109, 111].

Currently, in countries with moderate to high incidences, such as Portugal, mass screening such as the one observed in Japan and Korea is unwarranted [13]. Nevertheless, a gastroscopy in patients who are consulted due to other motives, such as a colorectal cancer screening by colonoscopy, has been proven to be cost-effective in our country [229, 230]. For that, the stratification of the population by GC risk might allow a personalized screening/surveillance, namely by targeted screening, optimization of surveillance intervals, and selection for chemoprevention, contributing to that cost-effectiveness. We reported an increased *PTGS2* mRNA expression in tumoral samples in our population, thus suggesting that the use of NSAIDs, namely aspirin, might represent a chemopreventive strategy for GC, particularly for those who will overexpress the COX-2 enzyme. Nevertheless, one should keep in mind that some genetic variants of *PTGS2* gene, namely the rs20417 polymorphism, might be associated with aspirin resistance [231].

6. CONCLUSION & FUTURE PERSPECTIVES

Overall, in this preliminary but original study in a Caucasian population, we observed associations between several genetic polymorphisms in four main players of the PGE_2 pathway, the *PTGS2*, *ABCC4, HPGD,* and *SLCO2A1* genes, and the susceptibility for GC development. Furthermore, we characterized their expression in this gastrointestinal model. To the best of our knowledge, and with the exception of *PTGS2* gene encoding the COX-2 protein, scarce to inexistent information was available for the PGE_2 pathway genes in gastric carcinogenesis. Due to a limited statistical power, future studies should warrant a higher number of participants, not only to clarify some of the observed statistical trends (displayed in Table S2), but also to be able to address the multiple testing correction. Concerning other future perspectives, we are interested in further investigating the functional repercussion of the addressed SNPs, due to the lack of information in the published literature and the fact that, even though most of them appear to not have an influence in mRNA expression, they could be regulators at protein level. Moreover, it would also be interesting to understand if there is a reciprocal regulation between COX-2, MRP4, 15-PGDH, and PGT. We are also interested in studying additional polymorphisms by real-time PCR, such as rs20417 in *PTGS2* gene, which, as previously mentioned, failed in genotyping; rs2555639, and rs2612656, both in *HPGD* gene, that failed to be converted to the Sequenom platform. These SNPs in the gene encoding the 15-PGDH protein have been associated with colorectal cancer development and progression [129, 131, 134, 232]. Therefore, we are intrigued to see if these polymorphisms might also play a role in gastric carcinogenesis. Concerning other techniques, immunohistochemistry has been a useful and effective tool to study protein expression in GC but there is also a lack of information regarding the expression of the four PGE_2 pathway proteins together in Caucasian populations, thus it would be interesting to explore that approach. Also, a role of COX-2 in early stages of gastric carcinogenesis has been suggested due to the overexpression found in pre-cancerous lesions of the stomach, such as metaplasia and noninvasive gastric dysplasia [84]. Therefore, we intend to reproduce this study in precursor lesions of GC and verify if we can identify individuals that may benefit from a personalized screening due to their distinct susceptibility profile. Additionally, we might be able to suggest that individuals that overexpress *PTGS2* gene should be targeted to a chemopreventive strategy.

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8. APPENDIX

Table S1. Genetic polymorphisms in *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genes and quality control results.

Gene	TagSNP	Other SNPs on the block	Genotype call rate (%)	Genotype concordance rate	HWE	Passed quality check?
PTGS2	rs5275	Candidate SNP	100	1.00	1.00	Yes
	rs20417	rs2066826 rs4648276 rs4648307	82.7	-	-	No
	rs689466	Candidate SNP	100	1.00	0.52	Yes
ABCC4	rs12867485	rs9561811 rs17189481	0	-	-	No
	rs1611822	rs1751015	99.9	1.00	0.85	Yes
	rs1628382	rs4148527 rs8001657 rs12584534	26.8	-	-	No
	rs1678354	rs1751059	100	1.00	<0.0001	No
	rs1678374	rs1751025	99.9	1.00	0.78	Yes
	rs1678386	rs9516530	100	1.00	0.51	Yes
	rs1678396	rs2766482	99.9	1.00	0.26	Yes
	rs1678405	rs2793821 rs6492768 rs7330933	98.9	1.00	0.42	Yes
	rs17268122	rs17268163	98.8	1.00	0.014	No
	rs1751027	rs1564351 rs4148487 rs17189390 rs17268170	100	1.00	0.23	Yes
	rs1751031	rs931111 rs1189444 rs1189451 rs1189452 rs1729747 rs2619312 rs5016378	99.9	1.00	0.20	Yes
	rs1751051	rs1751050	100	1.00	1.00	Yes
	rs2127295	rs1564355 rs1617785 rs1630807 rs1678363 rs1678394 rs1729748 rs2698243 rs2766481 rs3825415 rs6650282	99.6	1.00	0.40	Yes
	rs2274403	rs3864997 rs4148481	99.7	1.00	0.52	Yes
	rs2892713	rs12865305	99.9	1.00	0.14	Yes
	rs2892715	rs9561814	99.7	1.00	0.26	Yes
	rs3742106	rs4148544 rs4148549 rs4148551 rs7330196 rs9302039	100	1.00	0.92	Yes

SNP: Single Nucleotide Polymorphism; HWE: Hardy-Weinberg equilibrium.

Table S1 (cont.). Genetic polymorphisms in *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genes and quality control results.

Gene	TagSNP	Other SNPs on the block	Genotype call rate (%)	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs3742106	rs9524769				
	rs3782958	rs4148515 rs10508023	99.9	1.00	0.74	Yes
	rs4148421	rs9524864 rs9524873 rs10508017	99.9	1.00	1.00	Yes
	rs4148422	rs17300935	99.7	1.00	0.0022	No
	rs4148437	rs2892716 rs4148436 rs4148446 rs9556466 rs10508018	99.9	1.00	0.38	Yes
	rs4148476	rs4773843 rs9524822	100	1.00	0.13	Yes
	rs4612933	rs899494 rs899495 rs899496 rs1678403 rs1824911 rs1824913 rs1926657 rs3782965 rs4148465 rs4148469 rs4303338 rs4334136 rs4505186 rs4773854 rs4773855 rs7325019 rs7333234 rs7335147 rs7983336 rs7987653 rs7988494 rs9524831 rs9524833 rs9524845 rs9524856 rs12870204	100	1.00	1.00	Yes
	rs4771912	rs7981095	100	1.00	0.70	Yes
	rs6492763	rs10508024	100	1.00	0.34	Yes
	rs7993878	rs9302040 rs9302042 rs9302043 rs9556455 rs9561768 rs9561769 rs9590168 rs10219913	100	1.00	0.25	Yes

SNP: Single Nucleotide Polymorphism; HWE: Hardy-Weinberg equilibrium.

Table S1 (cont.). Genetic polymorphisms in *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genes and quality control results.

Gene	TagSNP	Other SNPs on the block	Genotype call rate (%)	Genotype concordance rate	HWE	Passed quality check?
ABCC4	rs8002180	rs4148424 rs4771910 rs7317112 rs7322318 rs8001475 rs9584288 rs9590228	99.9	1.00	0.43	Yes
	rs869951	rs871052 rs8001444	99.7	1.00	0.70	Yes
	rs9524821	rs9516532	99.9	1.00	0.42	Yes
	rs9590220	rs9590216 rs17235152	99.9	1.00	0.016	No
	rs9590222	rs12100301	64.4	-	-	No
HPGD	rs12500316	rs1863641 rs11722919	99.9	1.00	0.28	Yes
	rs1346271	singleton	99.7	1.00	0.065	Yes
	rs1426945	rs3756273	99.9	1.00	0.92	Yes
	rs1863642	rs2612659	100	1.00	0.23	Yes
	rs2303520	rs13127058	99.9	1.00	0.49	Yes
	rs2555632	rs3101255	99.9	1.00	0.43	Yes
	rs2612656	rs1816204 rs3846298	0	-	-	No
	rs8752	rs1426947 rs2612658 rs11133041 rs11724251	99.9	1.00	0.84	Yes
SLCO2A1	rs10935090	singleton	99.9	1.00	0.48	Yes
	rs1131598	singleton	99.7	1.00	0.26	Yes
	rs11915399	singleton	99.9	1.00	1.00	Yes
	rs4241362	rs4241361 rs4634113 rs6804798 rs9828294 rs9855403 rs9874493 rs9882333 rs11720811	99.9	1.00	0.42	Yes
	rs4241365	rs7653639	99.9	1.00	0.61	Yes
	rs4331673	rs11720843	100	1.00	0.87	Yes
	rs4854784	rs7636169	99.9	1.00	0.61	Yes
	rs6439448	rs2370512 rs3923835 rs4854768 rs4854769 rs34550074	99.9	1.00	0.88	Yes
	rs7340717	rs7340718	100	1.00	0.32	Yes
	rs7616492	rs10935089	99.9	1.00	0.69	Yes
	rs7625035	rs9822027	99.9	1.00	0.30	Yes

SNP: Single Nucleotide Polymorphism; HWE: Hardy-Weinberg equilibrium.

Table S1 (cont.). Genetic polymorphisms in *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genes and quality control results.

Gene	TagSNP	Other SNPs on the block	Genotype call rate (%)	Genotype concordance rate	HWE	Passed quality check?
SLCO2A1	rs7646392	rs4327389 rs4854777 rs5013525 rs7646298 rs7646473 rs12695600	99.9	1.00	0.29	Yes
	rs9820625	rs9836830 rs9917636 rs11709172 rs13083175	99.9	1.00	0.46	Yes
	rs9821091	rs6439450 rs7617777 rs7630191 rs9834727 rs9841380	99.9	1.00	0.44	Yes
	rs9834412	rs4854785 rs13067921	100	1.00	0.90	Yes

SNP: Single Nucleotide Polymorphism; HWE: Hardy-Weinberg equilibrium.

Table S2. Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
PTGS2											
rs5275	Codominant	TT	1.00	-	0.50	1.00	-	0.57	73.00	71.36-74.64	-
		TC	1.20	0.85-1.71		1.27	0.81-1.99		73.00	70.58-75.42	0.18
		CC	1.28	0.70-2.34		1.20	0.55-2.63		76.00	70.72-81.28	0.47
	Dominant	TT	1.00	-	0.25	1.00	-	0.29	73.00	71.36-74.64	-
		TC-CC	1.22	0.87-1.70		1.26	0.82-1.93		73.00	70.58-75.42	0.43
	Recessive	TT-TC	1.00	-	0.59	1.00	-	0.85	73.00	71.62-74.38	-
		CC	1.17	0.66-2.09		1.08	0.51-2.28		76.00	70.72-81.28	0.25
	Overdominant	TT-CC	1.00	-	0.39	1.00	-	0.34	73.00	71.32-74.68	-
		TC	1.16	0.83-1.62		1.24	0.80-1.90		73.00	71.58-75.42	0.12
	Log-additive	-	1.16	0.90-1.50	0.26	1.16	0.84-1.62	0.37	-		
rs689466	Codominant	AA	1.00	-	0.054	1.00	-	**0.021**	73.00	71.25-74.75	-
		AG	1.29	0.89-1.86		1.50	0.93-2.42		73.00	68.89-77.11	0.21
		GG	**2.33**	**1.10-4.92**		**3.40**	**1.29-8.97**		**70.00**	**62.49-77.52**	**0.008**
	Dominant	AA	1.00	-	0.056	1.00	-	**0.022**	73.00	71.25-74.75	-
		AG-GG	1.40	0.99-1.98		**1.69**	**1.08-2.65**		73.00	69.94-76.06	0.058
	Recessive	AA-AG	1.00	-	**0.046**	1.00	-	**0.027**	73.00	71.48-74.52	-
		GG	**2.15**	**1.03-4.49**		**2.98**	**1.14-7.74**		**70.00**	**62.49-77.52**	**0.011**
	Overdominant	AA-GG	1.00	-	0.30	1.00	-	0.19	73.00	71.46-74.54	-
		AG	1.21	0.85-1.74		1.37	0.86-2.19		73.00	68.89-77.11	0.38
	Log-additive	-	**1.40**	**1.06-1.86**	**0.021**	**1.66**	**1.15-2.40**	**0.007**	-		
ABCC4											
rs1611822	Codominant	CC	1.00	-	0.20	1.00	-	0.53	72.00	70.49-73.51	-
		CT	1.24	0.87-1.78		1.22	0.77-1.93		72.00	70.01-73.99	0.95
		TT	1.52	0.95-2.43		1.37	0.75-2.47		72.00	66.99-77.01	0.42
	Dominant	CC	1.00	-	0.12	1.00	-	0.30	72.00	70.49-73.51	-
		CT-TT	1.31	0.93-1.83		1.26	0.82-1.93		72.00	70.24-73.76	0.75
	Recessive	CC-CT	1.00	-	0.18	1.00	-	0.46	72.00	70.80-73.20	-
		TT	1.34	0.88-2.04		1.22	0.72-2.08		72.00	66.99-77.01	0.45
	Overdominant	CC-TT	1.00	-	0.62	1.00	-	0.66	72.00	70.55-73.45	-
		CT	1.08	0.79-1.49		1.10	0.73-1.65		72.00	70.01-73.99	0.78
	Log-additive	-	1.23	0.98-1.55	0.072	1.18	0.88-1.57	0.27	-		
rs1678374	Codominant	TT	1.00	-	0.076	1.00	-	0.063	72.00	69.48-74.52	-
		TC	0.82	0.58-1.15		1.04	0.67-1.63		72.00	69.74-74.26	0.47
		CC	**0.56**	**0.33-0.94**		**0.50**	**0.26-0.97**		73.00	71.00-75.00	0.57

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs1678374	Dominant	TT	1.00	-	0.09	1.00	-	0.55	72.00	69.48-74.52	-
		TC-CC	0.75	0.54-1.04		0.88	0.58-1.34		72.00	70.77-73.23	0.66
	Recessive	TT-TC	1.00	-	0.05	1.00	-	**0.019**	72.00	70.38-73.63	-
		CC	0.63	0.39-1.01		**0.49**	**0.26-0.91**		73.00	71.00-75.00	0.39
	Overdominant	TT-CC	1.00	-	0.30	1.00	-	0.28	72.00	70.46-73.54	-
		TC	1.21	0.85-1.74		1.25	0.83-1.89		72.00	69.74-74.26	0.31
	Log-additive	-	**0.77**	**0.60-0.97**	**0.027**	0.78	0.58-1.06	0.11	-		
rs1678386	Codominant	AA	1.00	-	0.82	1.00	-	0.34	72.00	70.04-73.96	-
		AC	0.90	0.64-1.26		0.75	0.49-1.16		72.00	70.48-73.52	0.80
		CC	0.98	0.56-1.72		1.13	0.55-2.33		71.00	58.59-83.41	0.87
	Dominant	AA	1.00	-	0.57	1.00	-	0.31	72.00	70.04-73.96	-
		AC-CC	0.91	0.66-1.26		0.81	0.54-1.22		72.00	70.06-73.94	0.89
	Recessive	AA-AC	1.00	-	0.93	1.00	-	0.50	72.00	70.91-73.09	-
		CC	1.03	0.59-1.77		1.28	0.63-2.58		71.00	58.59-83.41	0.75
	Overdominant	AA-CC	1.00	-	0.53	1.00	-	0.15	72.00	70.03-73.97	-
		AC	0.90	0.65-1.25		0.74	0.48-1.12		72.00	70.48-73.52	0.74
	Log-additive	-	0.95	0.75-1.22	0.70	0.92	0.67-1.27	0.63	-		
rs1678396	Codominant	TT	1.00	-	0.74	1.00	-	0.77	70.00	68.08-71.92	-
		TC	0.91	0.63-1.30		0.88	0.55-1.40		74.00	71.50-76.50	0.39
		CC	1.06	0.67-1.69		0.82	0.45-1.47		72.00	69.70-74.30	0.58
	Dominant	TT	1.00	-	0.75	1.00	-	0.50	70.00	68.08-71.92	-
		TC-CC	0.94	0.67-1.33		0.86	0.56-1.33		73.00	71.65-74.35	0.39
	Recessive	TT-TC	1.00	-	0.57	1.00	-	0.63	72.00	70.67-73.33	-
		CC	1.13	0.75-1.70		0.88	0.52-1.48		72.00	69.70-74.30	0.92
	Overdominant	TT-CC	1.00	-	0.46	1.00	-	0.81	71.00	69.65-72.35	-
		TC	0.89	0.64-1.22		0.95	0.63-1.43		74.00	71.50-76.50	0.39
	Log-additive	-	1.01	0.80-1.28	0.92	0.90	0.67-1.20	0.48	-		
rs1678405	Codominant	TT	1.00	-	0.052	1.00	-	**0.09**	72.00	70.40-73.60	-
		TC	0.73	0.52-1.02		0.81	0.52-1.25		72.00	69.60-74.40	0.58
		CC	**0.54**	**0.29-0.99**		**0.44**	**0.20-0.95**		74.00	67.60-80.40	0.68
	Dominant	TT	1.00	-	**0.027**	1.00	-	0.13	72.00	70.40-73.60	-
		TC-CC	**0.69**	**0.50-0.96**		0.73	0.48-1.10		72.00	70.18-73.82	0.70
	Recessive	TT-TC	1.00	-	0.11	1.00	-	**0.049**	72.00	70.58-73.42	-
		CC	0.63	0.35-1.13		**0.49**	**0.23-1.03**		74.00	67.60-80.40	0.58

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs1678405	Overdominant	TT-CC	1.00	-	0.21	1.00	-	0.76	72.00	70.46-73.54	-
		TC	0.81	0.59-1.12		0.94	0.62-1.42		72.00	69.60-74.40	0.48
	Log-additive	-	**0.73**	**0.57-0.94**	**0.015**	**0.72**	**0.52-0.99**	**0.041**	-		
rs1751027	Codominant	AA	1.00	-	0.096	1.00	-	0.66	72.00	70.19-73.81	-
		AG	1.30	0.86-1.96		1.27	0.73-2.20		72.00	71.05-72.95	0.51
		GG	6.82	0.70-66.05		1.64	0.12-22.90		73.00	66.60-79.40	0.96
	Dominant	AA	1.00	-	0.13	1.00	-	0.37	72.00	70.19-73.81	-
		AG-GG	1.37	0.92-2.05		1.28	0.75-2.20		72.00	71.00-73.00	0.52
	Recessive	AA-AG	1.00	-	0.076	1.00	-	0.73	72.00	70.58-73.42	-
		GG	6.51	0.67-62.91		1.57	0.11-22.03		73.00	66.60-79.40	1.00
	Overdominant	AA-GG	1.00	-	0.24	1.00	-	0.40	72.00	70.26-73.74	-
		AG	1.28	0.85-1.93		1.27	0.73-2.19		72.00	71.05-72.95	0.51
	Log-additive	-	1.42	0.97-2.08	0.074	1.27	0.76-2.12	0.36	-		
rs1751031	Codominant	AA	1.00	-	0.10	1.00	-	0.073	72.00	70.25-73.75	-
		AG	**0.69**	**0.48**		**0.61**	**0.39-0.95**		72.00	69.72-74.28	0.66
		GG	1.15	0.49-2.70		0.57	0.17-1.92		78.00	69.81-86.19	0.29
	Dominant	AA	1.00	-	0.068	1.00	-	**0.022**	72.00	70.25-73.75	-
		AG-GG	0.73	0.52-1.03		**0.60**	**0.39-0.94**		72.00	70.08-73.93	0.46
	Recessive	AA-AG	1.00	-	0.55	1.00	-	0.52	72.00	70.61-73.39	-
		GG	1.30	0.56-3.01		0.68	0.21-2.24		78.00	69.81-86.19	0.31
	Overdominant	AA-GG	1.00	-	**0.034**	1.00	-	**0.036**	72.00	70.28-73.72	-
		AG	**0.69**	**0.48-0.98**		**0.62**	**0.40-0.98**		72.00	69.72-74.28	0.79
	Log-additive	-	0.81	0.61-1.09	0.17	**0.65**	**0.44-0.96**	**0.028**	-		
rs1751051	Codominant	TT	1.00	-	0.058	1.00	-	0.22	71.00	69.28-72.72	-
		TA	**1.50**	**1.07-2.10**		1.39	0.90-2.14		73.00	71.46-74.54	0.96
		AA	1.36	0.77-2.41		1.61	0.80-3.21		71.00	65.65-76.35	0.54
	Dominant	TT	1.00	-	**0.018**	1.00	-	0.09	73.00	71.45-74.55	-
		TA-AA	**1.48**	**1.07-2.04**		1.43	0.94-2.16		71.00	69.28-72.72	0.86
	Recessive	TT-TA	1.00	-	0.71	1.00	-	0.35	72.00	70.85-73.15	-
		AA	1.11	0.65-1.90		1.37	0.71-2.64		71.00	65.65-76.35	0.49
	Overdominant	TT-AA	1.00	-	**0.032**	1.00	-	0.26	71.00	69.38-72.62	-
		TA	**1.42**	**1.03-1.95**		1.27	0.84-1.92		73.00	71.46-74.54	0.83
	Log-additive	-	**1.28**	**1.00-1.64**	**0.048**	1.31	0.96-1.78	0.088	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs2127295	Codominant	GG	1.00	-	0.39	1.00	-	0.30	73.00	71.01-74.99	-
		GA	0.94	0.64-1.36		1.10	0.67-1.81		71.00	69.16-72.84	0.61
		AA	1.24	0.79-1.95		1.55	0.86-2.79		72.00	69.64-74.36	0.50
	Dominant	GG	1.00	-	0.91	1.00	-	0.39	73.00	71.01-74.99	-
		GA-AA	1.02	0.72-1.45		1.22	0.77-1.95		72.00	70.40-73.60	0.52
	Recessive	GG-GA	1.00	-	0.18	1.00	-	0.14	72.00	70.51-73.49	-
		AA	1.30	0.89-1.90		1.46	0.89-2.37		72.00	69.64-74.36	0.49
	Overdominant	GG-AA	1.00	-	0.32	1.00	-	0.64	72.00	70.44-73.56	-
		GA	0.85	0.62-1.17		0.91	0.60-1.37		71.00	69.16-72.84	0.98
	Log-additive	-	1.10	0.88-1.39	0.39	1.24	0.92-1.67	0.15	-		
rs2274403	Codominant	AA	1.00	-	**0.016**	1.00	-	0.41	72.00	70.39-73.61	-
		AG	0.78	0.54-1.13		0.92	0.58-1.49		73.00	71.14-74.86	0.42
		GG	**0.51**	**0.32-0.81**		0.69	0.39-1.22		73.00	70.91-75.09	0.93
	Dominant	AA	1.00	-	**0.035**	1.00	-	0.44	72.00	70.39-73.61	-
		AG-GG	**0.69**	**0.48-0.97**		0.84	0.54-1.31		73.00	71.56-74.44	0.52
	Recessive	AA-AG	1.00	-	**0.01**	1.00	-	0.20	72.00	70.40-73.60	-
		GG	**0.60**	**0.40-0.89**		0.72	0.44-1.19		73.00	70.91-75.09	0.56
	Overdominant	AA-GG	1.00	-	0.80	1.00	-	0.70	72.00	70.75-73.25	-
		AG	1.04	0.76-1.43		1.08	0.72-1.63		73.00	71.14-74.86	0.31
	Log-additive	-	**0.72**	**0.57-0.91**	**0.0046**	0.84	0.63-1.11	0.22	-		
rs2892713	Codominant	CC	1.00	-	0.71	1.00	-	0.53	72.00	70.22-73.78	-
		CT	1.07	0.74-1.53		0.91	0.57-1.45		72.00	71.16-72.85	0.60
		TT	0.72	0.28-1.85		0.51	0.15-1.79		75.00	66.16-83.84	0.44
	Dominant	CC	1.00	-	0.89	1.00	-	0.50	72.00	70.22-73.78	-
		CT-TT	1.02	0.72-1.45		0.86	0.55-1.35		72.00	71.11-72.89	0.48
	Recessive	CC-CT	1.00	-	0.45	1.00	-	0.29	72.00	70.64-73.36	-
		TT	0.71	0.28-1.80		0.53	0.15-1.82		75.00	66.16-83.84	0.48
	Overdominant	CC-TT	1.00	-	0.66	1.00	-	0.79	72.00	70.17-73.83	-
		CT	1.08	0.76-1.55		0.94	0.59-1.49		72.00	71.16-72.85	0.66
	Log-additive	-	0.98	0.73-1.32	0.89	0.84	0.57-1.23	0.36	-		
rs2892715	Codominant	GG	1.00	-	0.52	1.00	-	0.73	72.00	70.56-73.44	-
		GA	1.07	0.75-1.53		1.18	0.75-1.85		73.00	71.20-74.80	0.56
		AA	0.82	0.51-1.33		0.99	0.54-1.81		72.00	69.81-74.19	0.76
	Dominant	GG	1.00	-	1.00	1.00	-	0.60	72.00	70.56-73.44	-
		GA-AA	1.00	0.72-1.40		1.12	0.73-1.72		73.00	71.58-74.42	0.67

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs2892715	Recessive	GG-GA	1.00	-	0.28	1.00	-	0.71	72.00	70.42-73.58	-
		AA	0.79	0.51-1.22		0.90	0.52-1.56		72.00	69.81-74.19	0.56
	Overdominant	GG-AA	1.00	-	0.42	1.00	-	0.43	72.00	70.79-73.21	-
		GA	1.14	0.83-1.57		1.18	0.78-1.78		73.00	71.20-74.80	0.42
	Log-additive	-	0.94	0.75-1.18	0.57	1.02	0.77-1.37	0.87	-		
rs3742106	Codominant	AA	1.00	-	0.17	1.00	-	0.65	72.00	69.49-74.51	-
		AC	1.12	0.79-1.58		1.13	0.72-1.76		72.00	70.22-73.79	0.85
		CC	0.70	0.42-1.18		0.85	0.44-1.63		73.00	68.95-77.05	0.92
	Dominant	AA	1.00	-	0.95	1.00	-	0.80	72.00	69.49-74.51	-
		AC-CC	1.01	0.72-1.41		1.06	0.69-1.62		72.00	70.78-73.22	0.86
	Recessive	AA-AC	1.00	-	0.075	1.00	-	0.44	72.00	70.39-73.61	-
		CC	0.66	0.41-1.06		0.79	0.43-1.44		73.00	68.95-77.05	0.96
	Overdominant	AA-CC	1.00	-	0.19	1.00	-	0.43	72.00	69.82-74.18	-
		AC	1.24	0.90-1.70		1.18	0.78-1.78		72.00	70.22-73.79	0.89
	Log-additive	-	0.90	0.71-1.14	0.38	0.97	0.71-1.31	0.83	-		
rs3782958	Codominant	GG	1.00	-	0.20	1.00	-	0.14	72.00	70.29-73.71	-
		GC	0.80	0.55-1.16		0.72	0.45-1.16		72.00	69.75-74.25	0.71
		CC	0.43	0.12-1.50		0.32	0.07-1.44		74.00	-	0.92
	Dominant	GG	1.00	-	0.14	1.00	-	0.096	72.00	70.29-73.71	-
		GC-CC	0.76	0.53-1.10		0.68	0.43-1.08		72.00	69.73-74.29	0.72
	Recessive	GG-GC	1.00	-	0.18	1.00	-	0.15	72.00	70.63-73.38	-
		CC	0.45	0.13-1.59		0.35	0.08-1.57		74.00	-	0.96
	Overdominant	GG-CC	1.00	-	0.29	1.00	-	0.23	72.00	70.28-73.72	-
		GC	0.82	0.57-1.19		0.75	0.47-1.20		72.00	69.75-74.25	0.70
	Log-additive	-	0.76	0.55-1.05	0.092	0.68	0.45-1.02	0.057	-		
rs4148421	Codominant	GG	1.00	-	0.66	1.00	-	0.27	71.00	67.11-74.89	-
		GA	0.90	0.61-1.31		0.69	0.43-1.12		72.00	70.81-73.19	0.81
		AA	1.07	0.69-1.66		0.92	0.52-1.63		72.00	69.24-74.76	0.62
	Dominant	GG	1.00	-	0.78	1.00	-	0.23	71.00	67.11-74.89	-
		GA-AA	0.95	0.67-1.35		0.76	0.48-1.19		72.00	70.84-73.16	0.71
	Recessive	GG-GA	1.00	-	0.47	1.00	-	0.55	72.00	70.54-73.46	-
		AA	1.15	0.79-1.67		1.16	0.71-1.89		72.00	69.24-74.76	0.63
	Overdominant	GG-AA	1.00	-	0.39	1.00	-	0.11	72.00	69.71-74.29	-
		GA	0.87	0.63-1.20		0.72	0.47-1.08		72.00	70.81-73.19	0.93
	Log-additive	-	1.03	0.82-1.29	0.81	0.94	0.70-1.26	0.68	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant ($P<0.05$).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs4148437	Codominant	TT	1.00	-	0.67	1.00	-	0.87	72.00	70.47-73.53	-
		TC	1.08	0.76-1.52		1.09	0.70-1.71		73.00	71.10-74.91	0.38
		CC	0.86	0.52-1.43		0.95	0.51-1.79		72.00	66.67-77.33	0.65
	Dominant	TT	1.00	-	0.90	1.00	-	0.79	72.00	70.47-73.53	-
		TC-CC	1.02	0.74-1.42		1.06	0.70-1.61		73.00	71.55-74.45	0.52
	Recessive	TT-TC	1.00	-	0.43	1.00	-	0.74	72.00	70.47-73.53	-
		CC	0.83	0.52-1.32		0.91	0.51-1.63		72.00	66.67-77.33	0.45
	Overdominant	TT-CC	1.00	-	0.50	1.00	-	0.62	72.00	70.67-73.33	-
		TC	1.12	0.81-1.54		1.11	0.73-1.67		73.00	71.10-74.91	0.27
	Log-additive	-	0.96	0.76-1.22	0.76	1.00	0.75-1.35	0.98	-		
rs4148476	Codominant	TT	1.00	-	0.81	1.00	-	0.44	72.00	70.67-73.34	-
		TG	1.12	0.78-1.61		1.27	0.79-2.05		74.00	70.85-77.15	0.57
		GG	0.98	0.42-2.31		1.66	0.58-4.77		71.00	55.55-86.46	0.61
	Dominant	TT	1.00	-	0.57	1.00	-	0.23	72.00	70.67-73.34	-
		TG-GG	1.11	0.78-1.56		1.32	0.84-2.07		73.00	69.73-76.27	0.69
	Recessive	TT-TG	1.00	-	0.91	1.00	-	0.42	72.00	70.88-73.12	-
		GG	0.95	0.41-2.22		1.56	0.55-4.42		71.00	55.55-86.46	0.57
	Overdominant	TT-GG	1.00	-	0.52	1.00	-	0.37	72.00	70.72-73.28	-
		TG	1.12	0.79-1.61		1.24	0.78-1.98		74.00	70.85-77.15	0.53
	Log-additive	-	1.07	0.80-1.42	0.66	1.28	0.88-1.86	0.20	-		
rs4612933	Codominant	CC	1.00	-	0.45	1.00	-	0.31	72.00	70.39-73.61	-
		CT	0.81	0.56-1.16		0.73	0.46-1.15		72.00	69.83-74.17	0.44
		TT	1.13	0.51-2.50		1.31	0.48-3.61		67.00	58.62-75.38	0.68
	Dominant	CC	1.00	-	0.32	1.00	-	0.27	72.00	70.39-73.61	-
		CT-TT	0.84	0.60-1.18		0.78	0.51-1.21		72.00	70.00-74.00	0.56
	Recessive	CC-CT	1.00	-	0.65	1.00	-	0.48	72.00	70.60-73.40	-
		TT	1.20	0.54-2.64		1.44	0.53-3.94		67.00	58.62-75.38	0.57
	Overdominant	CC-TT	1.00	-	0.22	1.00	-	0.15	72.00	70.42-73.58	-
		CT	0.80	0.56-0.14		0.72	0.45-1.13		72.00	69.83-74.17	0.40
	Log-additive	-	0.91	0.68-1.21	0.50	0.88	0.61-1.27	0.50	-		
rs4771912	Codominant	AA	1.00	-	0.52	1.00	-	0.20	72.00	70.79-73.21	-
		AG	0.84	0.57-1.24		0.64	0.38-1.08		72.00	69.45-74.55	0.19
		GG	0.61	0.17-2.26		0.58	0.14-2.46		70.00	-	0.71
	Dominant	AA	1.00	-	0.30	1.00	-	0.071	72.00	70.79-73.21	-
		AG-GG	0.82	0.56-1.20		0.64	0.39-1.05		72.00	70.49-73.51	0.24

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs4771912	Recessive	AA-AG	1.00	-	0.48	1.00	-	0.53	72.00	70.87-73.13	-
		GG	0.64	0.17-2.34		0.64	0.15-2.70		70.00	-	0.58
	Overdominant	AA-GG	1.00	-	0.40	1.00	-	0.10	72.00	70.28-73.72	-
		AG	0.85	0.57-1.25		0.65	0.39-1.09		72.00	69.45-74.55	0.18
	Log-additive	-	0.82	0.58-1.16	0.26	0.68	0.44-1.05	0.077	-		
rs6492763	Codominant	TT	1.00	-	0.23	1.00	-	0.26	72.00	70.55-73.45	-
		TC	0.75	0.53-1.06		0.79	0.51-1.24		72.00	69.89-74.11	0.66
		CC	0.74	0.45-1.23		0.60	0.31-1.13		72.00	64.37-79.63	0.88
	Dominant	TT	1.00	-	0.084	1.00	-	0.17	72.00	70.55-73.45	-
		TC-CC	0.75	0.54-1.04		0.74	0.49-1.13		72.00	69.94-74.06	0.73
	Recessive	TT-TC	1.00	-	0.56	1.00	-	0.19	72.00	70.61-73.39	-
		CC	0.87	0.55-1.39		0.68	0.37-1.23		72.00	64.37-79.63	0.71
	Overdominant	TT-CC	1.00	-	0.20	1.00	-	0.68	72.00	70.48-73.52	-
		TC	0.81	0.59-1.12		0.92	0.61-1.38		72.00	69.89-74.11	0.55
	Log-additive	-	0.83	0.66-1.05	0.12	0.78	0.58-1.05	0.10	-		
rs7993878	Codominant	GG	1.00	-	0.40	1.00	-	0.18	72.00	70.54-73.46	-
		GA	0.84	0.57-1.25		0.67	0.40-1.13		76.00	73.59-78.42	0.29
		AA	0.51	0.14-1.83		0.42	0.09-1.97		68.00	51.28-84.72	0.84
	Dominant	GG	1.00	-	0.26	1.00	-	0.079	72.00	70.54-73.46	-
		GA-AA	0.81	0.55-1.18		0.64	0.39-1.06		76.00	73.52-78.48	0.29
	Recessive	GG-GA	1.00	-	0.30	1.00	-	0.29	72.00	70.63-73.37	-
		AA	0.53	0.15-1.90		0.46	0.10-2.14		68.00	51.28-84.72	0.90
	Overdominant	GG-AA	1.00	-	0.42	1.00	-	0.15	72.00	70.53-73.47	-
		GA	0.85	0.57-1.26		0.69	0.41-1.15		76.00	73.59-78.42	0.30
	Log-additive	-	0.80	0.57-1.13	0.20	0.66	0.42-1.04	0.064	-		
rs8002180	Codominant	TT	1.00	-	0.79	1.00	-	0.83	72.00	70.70-73.30	-
		TC	1.10	0.79-1.53		0.92	0.60-1.41		72.00	70.27-73.73	0.31
		CC	0.92	0.51-1.67		0.80	0.37-1.74		79.00	72.08-85.92	0.53
	Dominant	TT	1.00	-	0.70	1.00	-	0.60	72.00	70.70-73.30	-
		TC-CC	1.07	0.77-1.47		0.90	0.59-1.35		72.00	69.90-74.10	0.52
	Recessive	TT-TC	1.00	-	0.68	1.00	-	0.64	72.00	70.67-73.33	-
		CC	0.89	0.50-1.58		0.84	0.40-1.77		79.00	72.08-85.92	0.34
	Overdominant	TT-CC	1.00	-	0.53	1.00	-	0.79	72.00	70.62-73.38	-
		TC	1.11	0.80-1.54		0.95	0.62-1.43		72.00	70.27-73.73	0.21
	Log-additive	-	1.02	0.79-1.30	0.90	0.90	0.66-1.25	0.54	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant ($P<0.05$).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs869951	Codominant	GG	1.00	-	0.33	1.00	-	0.43	72.00	69.52-74.48	-
		GC	1.28	0.90-1.84		1.35	0.85-2.13		72.00	70.61-73.39	0.89
		CC	1.03	0.63-1.68		1.28	0.69-2.38		71.00	69.51-72.50	0.71
	Dominant	GG	1.00	-	0.26	1.00	-	0.20	72.00	69.52-74.48	-
		GC-CC	1.22	0.87-1.71		1.33	0.86-2.06		72.00	70.34-73.66	0.79
	Recessive	GG-GC	1.00	-	0.58	1.00	-	0.79	72.00	70.78-73.22	-
		CC	0.88	0.57-1.37		1.08	0.62-1.87		71.00	69.51-72.50	0.64
	Overdominant	GG-CC	1.00	-	0.14	1.00	-	0.30	72.00	70.39-73.61	-
		GC	1.27	0.92-1.75		1.24	0.82-1.87		72.00	70.61-73.39	0.94
	Log-additive	-	1.06	0.84-1.33	0.63	1.17	0.87-1.57	0.30	-		
rs9524821	Codominant	GG	1.00	-	0.15	1.00	-	0.33	72.00	70.08-73.92	-
		GA	1.05	0.74-1.49		1.22	0.78-1.92		72.00	70.15-73.85	0.48
		AA	1.56	0.98-2.49		1.57	0.85-2.89		71.00	67.01-74.99	0.86
	Dominant	GG	1.00	-	0.35	1.00	-	0.21	72.00	70.08-73.92	-
		GA-AA	1.17	0.84-1.62		1.31	0.86-1.99		72.00	70.29-73.71	0.63
	Recessive	GG-GA	1.00	-	0.056	1.00	-	0.23	72.00	70.88-73.13	-
		AA	1.53	0.99-2.34		1.42	0.81-2.48		71.00	67.01-74.99	0.62
	Overdominant	GG-AA	1.00	-	0.64	1.00	-	0.71	72.00	69.99-74.01	-
		GA	0.93	0.67-1.28		1.08	0.72-1.63		72.00	70.15-73.85	0.37
	Log-additive	-	1.21	0.96-1.51	0.10	1.25	0.93-1.67	0.14	-		
HPGD											
rs12500316	Codominant	CC	1.00	-	0.18	1.00	-	0.51	72.00	70.65-73.35	-
		CT	0.74	0.53-1.04		0.78	0.51-1.21		72.00	68.82-75.18	0.47
		TT	1.12	0.57-2.18		1.06	0.44-2.52		72.00	69.44-74.56	0.44
	Dominant	CC	1.00	-	0.15	1.00	-	0.34	72.00	70.65-73.35	-
		CT-TT	0.79	0.57-1.09		0.82	0.54-1.23		72.00	70.01-73.99	0.35
	Recessive	CC-CT	1.00	-	0.51	1.00	-	0.72	72.00	70.51-73.49	-
		TT	1.25	0.65-2.41		1.17	0.50-2.74		72.00	69.44-74.56	0.51
	Overdominant	CC-TT	1.00	-	0.069	1.00	-	0.25	72.00	70.81-73.19	-
		CT	0.73	0.53-1.03		0.78	0.51-1.19		72.00	68.82-75.18	0.53
	Log-additive	-	0.88	0.68-1.15	0.35	0.89	0.64-1.25	0.52	-		
rs1346271	Codominant	GG	1.00	-	**0.035**	1.00	-	0.30	73.00	70.85-75.15	-
		GC	**0.68**	**0.48-0.96**		0.71	0.45-1.11		72.00	70.06-73.94	0.48
		CC	1.12	0.69-1.82		0.93	0.50-1.74		71.00	68.85-73.15	0.77

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs1346271	Dominant	GG	1.00	-	0.11	1.00	-	0.20	73.00	70.85-75.15	-
		GC-CC	0.77	0.55-1.06		0.76	0.50-1.15		72.00	70.63-73.37	0.54
	Recessive	GG-GC	1.00	-	0.16	1.00	-	0.69	72.00	70.83-73.17	-
		CC	1.38	0.88-2.17		1.12	0.63-2.01		71.00	68.85-73.15	0.92
	Overdominant	GG-CC	1.00	-	**0.011**	1.00	-	0.13	72.00	70.32-73.68	-
		GC	**0.66**	**0.48-0.91**		0.73	0.48-1.10		72.00	70.06-73.94	0.50
	Log-additive	-	0.95	0.75-1.20	0.66	0.90	0.66-1.21	0.48	-		
rs1426945	Codominant	GG	1.00	-	0.32	1.00	-	0.59	72.00	69.94-74.06	-
		GA	0.82	0.57-1.17		0.97	0.62-1.54		72.00	69.43-74.57	0.89
		AA	1.11	0.70-1.75		1.30	0.72-2.35		72.00	69.41-74.59	0.40
	Dominant	GG	1.00	-	0.49	1.00	-	0.80	72.00	69.94-74.06	-
		GA-AA	0.89	0.64-1.24		1.06	0.69-1.62		72.00	70.15-73.85	0.68
	Recessive	GG-GA	1.00	-	0.31	1.00	-	0.31	72.00	70.38-73.62	-
		AA	1.24	0.82-1.88		1.32	0.78-2.25		72.00	69.41-74.59	0.44
	Overdominant	GG-AA	1.00	-	0.15	1.00	-	0.59	72.00	70.38-73.62	-
		GA	0.79	0.57-1.09		0.89	0.59-1.35		72.00	69.43-74.57	0.85
	Log-additive	-	1.01	0.81-1.27	0.93	1.11	0.83-1.49	0.47	-		
rs1863642	Codominant	GG	1.00	-	0.18	1.00	-	0.56	73.00	71.38-74.62	-
		GT	0.74	0.53-1.03		0.87	0.57-1.34		72.00	69.75-74.25	0.15
		TT	1.03	0.57-1.85		1.31	0.62-2.77		70.00	67.19-72.81	0.10
	Dominant	GG	1.00	-	0.13	1.00	-	0.74	73.00	71.38-74.62	-
		GT-TT	0.78	0.57-1.08		0.93	0.62-1.40		72.00	70.22-73.78	0.07
	Recessive	GG-GT	1.00	-	0.58	1.00	-	0.38	72.00	70.74-73.26	-
		TT	1.17	0.67-2.08		1.39	0.67-2.87		70.00	67.19-72.81	0.16
	Overdominant	GG-TT	1.00	-	0.064	1.00	-	0.40	72.00	70.68-73.32	-
		GT	0.74	0.53-1.02		0.84	0.55-1.27		72.00	69.75-74.25	0.25
	Log-additive	-	0.88	0.69-1.14	0.34	1.02	0.74-1.41	0.90	-		
rs2303520	Codominant	GG	1.00	-	**0.037**	1.00	-	0.065	72.00	70.89-73.11	-
		GA	**1.48**	**1.04-2.09**		**1.61**	**1.02-2.54**		72.00	69.12-74.88	0.83
		AA	0.51	0.14-1.79		0.51	0.11-2.34		69.00	64.20-73.80	0.61
	Dominant	GG	1.00	-	0.066	1.00	-	0.086	72.00	70.89-73.11	-
		GA-AA	1.38	0.98-1.93		1.47	0.95-2.29		72.00	69.05-74.96	0.92
	Recessive	GG-GA	1.00	-	0.18	1.00	-	0.26	72.00	70.63-73.37	-
		AA	0.45	0.13-1.59		0.45	0.10-2.04		69.00	64.20-73.80	0.61

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs2303520	Overdominant	GG-AA	1.00	-	**0.021**	1.00	-	**0.031**	72.00	70.87-73.13	-
		GA	**1.51**	**1.07-2.13**		**1.65**	**1.05-2.59**		72.00	69.12-74.88	0.81
	Log-additive	-	1.21	0.90-1.64	0.21	1.26	0.86-1.84	0.24	-		
rs2555632	Codominant	TT	1.00	-	0.45	1.00	-	0.16	73.00	71.62-74.38	-
		TC	1.00	0.71-1.41		1.29	0.83-2.00		72.00	69.22-74.78	0.58
		CC	1.57	0.78-3.14		2.19	0.90-5.30		**70.00**	**66.67-73.33**	**0.019**
	Dominant	TT	1.00	-	0.71	1.00	-	0.13	73.00	71.62-74.38	-
		TC-CC	1.06	0.77-1.47		1.39	0.91-2.10		71.00	68.72-73.28	0.26
	Recessive	TT-TC	1.00	-	0.20	1.00	-	0.12	72.00	70.73-73.27	-
		CC	1.57	0.79-3.10		1.99	0.84-4.73		**70.00**	**66.67-73.33**	**0.027**
	Overdominant	TT-CC	1.00	-	0.83	1.00	-	0.41	72.00	70.61-73.39	-
		TC	0.96	0.69-1.34		1.20	0.78-1.85		72.00	69.22-74.78	0.82
	Log-additive	-	1.11	0.85-1.45	0.43	1.38	0.98-1.94	0.066	-		
rs8752	Codominant	AA	1.00	-	0.32	1.00	-	0.30	72.00	70.13-73.87	-
		AG	1.28	0.90-1.80		1.39	0.90-2.17		72.00	70.32-73.68	0.60
		GG	0.99	0.59-1.68		1.39	0.71-2.72		72.00	66.96-77.04	0.31
	Dominant	AA	1.00	-	0.25	1.00	-	0.12	72.00	70.13-73.87	-
		AG-GG	1.21	0.87-1.68		1.39	0.91-2.12		72.00	70.45-73.55	0.46
	Recessive	AA-AG	1.00	-	0.56	1.00	-	0.64	72.00	70.53-73.47	-
		GG	0.87	0.53-1.41		1.16	0.62-2.16		72.00	66.96-77.04	0.41
	Overdominant	AA-GG	1.00	-	0.13	1.00	-	0.22	72.00	70.42-73.58	-
		AG	1.28	0.93-1.76		1.29	0.86-1.94		72.00	70.32-73.68	0.83
	Log-additive	-	1.07	0.84-1.35	0.58	1.24	0.91-1.68	0.17	-		
SLCO2A1											
rs10935090	Codominant	CC	1.00	-	0.13	1.00	-	**0.026**	73.00	71.81-74.19	-
		CT	1.40	0.95-2.06		1.46	0.90-2.39		**70.00**	**67.56-72.44**	**0.034**
		TT	2.00	0.66-6.04		**4.68**	**1.32-16.61**		**62.00**	**59.61-64.39**	**<0.001**
	Dominant	CC	1.00	-	0.054	1.00	-	**0.038**	73.00	71.81-74.19	-
		CT-TT	1.44	1.00-2.09		**1.65**	**1.03-2.63**		**70.00**	**67.93-72.07**	**0.007**
	Recessive	CC-CT	1.00	-	0.028	1.00	-	**0.026**	72.00	70.87-73.14	-
		TT	1.86	0.62-5.59		**4.30**	**1.22-15.16**		**62.00**	**59.61-64.39**	**<0.001**
	Overdominant	CC-TT	1.00	-	0.11	1.00	-	0.19	73.00	71.807-74.193	-
		CT	1.37	0.94-2.02		1.39	0.86-2.27		70.00	67.56-72.44	0.057
	Log-additive	-	**1.40**	**1.01-1.95**	**0.044**	**1.69**	**1.12-2.53**	**0.012**	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant ($P<0.05$).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs1131598	Codominant	AA	1.00	-	0.67	1.00	-	0.59	72.00	70.55-73.45	-
		AG	0.90	0.64-1.25		0.81	0.52-1.24		73.00	71.22-74.78	0.86
		GG	0.74	0.32-1.71		1.07	0.40-2.91		69.00	62.82-75.18	0.54
	Dominant	AA	1.00	-	0.44	1.00	-	0.38	72.00	70.55-73.45	-
		AG-GG	0.88	0.64-1.22		0.83	0.55-1.26		72.00	70.17-73.84	0.96
	Recessive	AA-AG	1.00	-	0.53	1.00	-	0.76	72.00	70.88-73.12	-
		GG	0.77	0.34-1.77		1.17	0.44-3.12		69.00	62.82-75.18	0.46
	Overdominant	AA-GG	1.00	-	0.59	1.00	-	0.31	72.00	70.39-73.61	-
		AG	0.91	0.66-1.27		0.80	0.52-1.22		73.00	71.22-74.78	0.77
	Log-additive	-	0.88	0.67-1.17	0.38	0.89	0.62-1.27	0.52	-		
rs11915399	Codominant	CC	1.00	-	0.72	1.00	-	0.12	72.00	70.27-73.73	-
		CT	0.87	0.60-1.24		**0.61**	**0.38-0.99**		73.00	71.47-74.53	0.13
		TT	0.88	0.33-2.33		0.75	0.22-2.63		74.00	59.93-88.07	0.52
	Dominant	CC	1.00	-	0.42	1.00	-	**0.043**	72.00	70.27-73.73	-
		CT-TT	0.87	0.61-1.23		**0.62**	**0.39-0.99**		73.00	71.53-74.47	0.11
	Recessive	CC-CT	1.00	-	0.86	1.00	-	0.81	72.00	70.64-73.37	-
		TT	0.91	0.35-2.41		0.86	0.25-2.96		74.00	59.93-88.07	0.59
	Overdominant	CC-TT	1.00	-	0.45	1.00	-	**0.045**	72.00	70.19-73.81	-
		CT	0.87	0.61-1.25		**0.62**	**0.38-1.00**		73.00	71.47-74.53	0.16
	Log-additive	-	0.89	0.65-1.21	0.45	0.69	0.46-1.03	0.065	-		
rs4241362	Codominant	TT	1.00	-	0.97	1.00	-	0.49	72.00	70.65-73.35	-
		TC	0.97	0.67-1.39		1.13	0.71-1.80		73.00	68.67-77.63	0.82
		CC	0.93	0.37-2.30		1.86	0.65-5.31		**65.00**	**62.94-67.06**	**0.019**
	Dominant	TT	1.00	-	0.83	1.00	-	0.42	72.00	70.65-73.35	-
		TC-CC	0.96	0.68-1.36		1.20	0.77-1.87		72.00	68.34-75.67	0.82
	Recessive	TT-TC	1.00	-	0.88	1.00	-	0.29	72.00	70.87-73.13	-
		CC	0.93	0.38-2.30		1.79	0.63-5.07		**65.00**	**62.94-67.06**	**0.024**
	Overdominant	TT-CC	1.00	-	0.87	1.00	-	0.70	72.00	70.64-73.36	-
		TC	0.97	0.68-1.39		1.10	0.69-1.74		73.00	68.67-77.63	0.70
	Log-additive	-	0.97	0.72-1.30	0.82	1.22	0.84-1.78	0.29	-		
rs4241365	Codominant	TT	1.00	-	0.89	1.00	-	0.53	72.00	70.71-73.30	-
		TC	1.08	0.77-1.51		1.10	0.71-1.71		72.00	69.77-74.23	0.58
		CC	1.11	0.56-2.17		1.65	0.68-3.99		74.00	67.61-80.39	0.61
	Dominant	TT	1.00	-	0.63	1.00	-	0.47	72.00	70.71-73.30	-
		TC-CC	1.08	0.78-1.50		1.17	0.77-1.77		72.00	69.67-74.33	0.71

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs4241365	Recessive	TT-TC	1.00	-	0.83	1.00	-	0.30	72.00	70.65-73.35	-
		CC	1.07	0.55-2.08		1.59	0.67-3.78		74.00	67.61-80.39	0.56
	Overdominant	TT-CC	1.00	-	0.69	1.00	-	0.80	72.00	69.77-74.23	-
		TC	1.07	0.77-1.49		1.06	0.69-1.62		72.00	69.77-74.23	0.51
	Log-additive	-	1.07	0.82-1.38	0.63	1.19	0.84-1.67	0.32	-		
rs4331673	Codominant	CC	1.00	-	0.60	1.00	-	0.19	72.00	70.80-73.20	-
		CA	0.88	0.61-1.27		0.92	0.58-1.48		72.00	68.55-75.45	0.48
		AA	1.35	0.57-3.18		2.61	0.92-7.41		65.00	62.64-67.36	0.32
	Dominant	CC	1.00	-	0.67	1.00	-	0.84	72.00	70.80-73.20	-
		CA-AA	0.93	0.65-1.31		1.05	0.67-1.64		72.00	68.42-75.58	0.69
	Recessive	CC-CA	1.00	-	0.45	1.00	-	0.072	72.00	70.63-73.37	-
		AA	1.39	0.59-3.27		2.67	0.95-7.52		65.00	62.64-67.36	0.33
	Overdominant	CC-AA	1.00	-	0.45	1.00	-	0.59	72.00	70.53-73.48	-
		CA	0.87	0.61-1.25		0.88	0.55-1.40		72.00	68.55-75.45	0.44
	Log-additive	-	0.98	0.73-1.32	0.91	1.17	0.80-1.70	0.43	-		
rs4854784	Codominant	GG	1.00	-	0.58	1.00	-	0.73	72.00	70.10-73.90	-
		GA	0.98	0.70-1.37		1.06	0.69		72.00	70.64-73.36	0.27
		AA	0.75	0.43-1.31		1.32	0.67-2.62		67.00	65.21-68.80	0.10
	Dominant	GG	1.00	-	0.66	1.00	-	0.62	72.00	70.10-73.90	-
		GA-AA	0.93	0.68-1.28		1.11	0.74-1.68		72.00	70.26-73.74	0.59
	Recessive	GG-GA	1.00	-	0.30	1.00	-	0.46	72.00	70.84-73.16	-
		AA	0.76	0.44-1.30		1.28	0.67-2.46		67.00	65.21-68.80	0.071
	Overdominant	GG-AA	1.00	-	0.85	1.00	-	0.97	72.00	69.98-74.02	-
		GA	1.03	0.75-1.42		1.01	0.67-1.53		72.00	70.64-73.36	0.16
	Log-additive	-	0.91	0.71-1.15	0.42	1.12	0.82-1.52	0.47	-		
rs6439448	Codominant	CC	1.00	-	0.31	1.00	-	0.48	72.00	70.30-73.70	-
		CA	1.14	0.81-1.61		1.10	0.70-1.72		72.00	69.71-74.29	0.54
		AA	0.52	0.17-1.56		0.51	0.14-1.82		70.00	64.09-75.91	0.92
	Dominant	CC	1.00	-	0.67	1.00	-	0.91	72.00	70.30-73.70	-
		CA-AA	1.08	0.77-1.50		1.03	0.67-1.58		72.00	69.99-74.01	0.56
	Recessive	CC-CA	1.00	-	0.18	1.00	-	0.25	72.00	70.63-73.37	-
		AA	0.49	0.16-1.49		0.49	0.14-1.75		70.00	64.09-75.91	0.91
	Overdominant	CC-AA	1.00	-	0.36	1.00	-	0.58	72.00	70.28-73.73	-
		CA	1.17	0.83-1.65		1.13	0.73-1.76		72.00	69.71-74.29	0.53
	Log-additive	-	1.00	0.74-1.34	0.98	0.95	0.65-1.38	0.79	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs7340717	Codominant	GG	1.00	-	0.30	1.00	-	0.37	72.00	70.34-73.66	-
		GT	1.15	0.82-1.62		1.23	0.79-1.91		72.00	69.69-74.31	0.53
		TT	0.77	0.45-1.32		0.80	0.41-1.56		74.00	70.51-77.49	0.63
	Dominant	GG	1.00	-	0.72	1.00	-	0.59	72.00	70.34-73.66	-
		GT-TT	1.06	0.77-1.47		1.12	0.74-1.69		72.00	70.18-73.82	0.49
	Recessive	GG-GT	1.00	-	0.19	1.00	-	0.29	72.00	70.62-73.38	-
		TT	0.72	0.43-1.19		0.72	0.38-1.34		74.00	70.51-77.49	0.80
	Overdominant	GG-TT	1.00	-	0.22	1.00	-	0.22	72.00	70.31-73.70	-
		GT	1.22	0.89-1.68		1.30	0.86-1.96		72.00	69.69-74.31	0.60
	Log-additive	-	0.96	0.76-1.21	0.71	0.98	0.73-1.32	0.90	-		
rs7616492	Codominant	GG	1.00	-	0.83	1.00	-	0.74	72.00	69.60-74.40	-
		GA	1.08	0.77-1.52		1.06	0.69-1.65		72.00	69.76-74.24	0.94
		AA	1.15	0.70-1.89		0.82	0.43-1.59		72.00	69.58-74.42	0.34
	Dominant	GG	1.00	-	0.58	1.00	-	0.98	72.00	69.60-74.40	-
		GA-AA	1.10	0.79-1.52		1.01	0.67-1.52		72.00	70.73-73.27	0.63
	Recessive	GG-GA	1.00	-	0.68	1.00	-	0.47	72.00	69.58-74.42	-
		AA	1.10	0.69-1.75		0.80	0.43-1.48		72.00	69.58-74.42	0.36
	Overdominant	GG-AA	1.00	-	0.79	1.00	-	0.61	72.00	70.26-73.74	-
		GA	1.05	0.76-1.44		1.11	0.74-1.68		72.00	69.76-74.24	0.85
	Log-additive	-	1.07	0.85-1.35	0.55	0.95	0.70-1.28	0.74	-		
rs7625035	Codominant	AA	1.00	-	0.29	1.00	-	0.48	72.00	70.10-73.90	-
		AG	0.85	0.61-1.20		0.91	0.59-1.40		72.00	70.08-73.92	0.66
		GG	1.49	0.74-2.98		1.57	0.67-3.71		72.00	66.87-77.13	0.19
	Dominant	AA	1.00	-	0.62	1.00	-	0.94	72.00	70.10-73.90	-
		AG-GG	0.92	0.67-1.27		0.98	0.65-1.49		72.00	70.40-73.60	0.43
	Recessive	AA-AG	1.00	-	0.20	1.00	-	0.26	72.00	70.55-73.45	-
		GG	1.58	0.80-3.12		1.63	0.70-3.79		72.00	66.87-77.13	0.20
	Overdominant	AA-GG	1.00	-	0.26	1.00	-	0.52	72.00	70.08-73.92	-
		AG	0.83	0.59-1.16		0.87	0.57-1.33		72.00	70.08-73.92	0.83
	Log-additive	-	1.01	0.77-1.32	0.94	1.07	0.76-1.49	0.70	-		
rs7646392	Codominant	CC	1.00	-	0.63	1.00	-	0.90	72.00	69.68-74.32	-
		CT	0.87	0.61-1.23		0.90	0.57-1.41		72.00	70.42-73.58	0.64
		TT	0.82	0.51-1.31		0.94	0.52-1.71		72.00	67.51-76.50	0.87
	Dominant	CC	1.00	-	0.35	1.00	-	0.66	72.00	69.68-74.32	-
		CT-TT	0.86	0.62-1.19		0.91	0.60-1.38		72.00	70.69-73.31	0.65

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant ($P<0.05$).

Table S2 (cont.). Genotype risk estimates for the involvement of *PTGS2*, *ABCC4*, *HPGD*, and *SLCO2A1* genetic variants in gastric cancer onset and estimated age at diagnosis.

SNP	Model	Genotype	Univariate analysis			Multivariate analysis*			Age at diagnosis		
			OR	95% CI	P value	aOR	95% CI	P value	Median (years)	95% CI	P value
rs7646392	Recessive	CC-CT	1.00	-	0.57	1.00	-	1.00	72.00	70.70-73.30	-
		TT	0.88	0.57-1.36		1.00	0.58-1.73		72.00	67.51-76.50	0.93
	Overdominant	CC-TT	1.00	-	0.62	1.00	-	0.67	72.00	70.08-73.92	-
		CT	0.92	0.67-1.27		0.91	0.60-1.38		72.00	70.42-73.58	0.61
	Log-additive	-	0.90	0.72-1.13	0.35	0.96	0.72-1.28	0.76	-		
rs9820625	Codominant	AA	1.00	-	1.00	1.00	-	0.84	72.00	69.85-74.15	-
		AC	0.99	0.68-1.43		1.06	0.66-1.71		72.00	70.12-73.88	0.80
		CC	0.99	0.63-1.55		1.19	0.67-2.09		72.00	68.89-75.11	0.31
	Dominant	AA	1.00	-	0.94	1.00	-	0.68	72.00	69.85-74.15	-
		AC-CC	0.99	0.70-1.40		1.10	0.70-1.72		72.00	70.32-73.68	0.86
	Recessive	AA-AC	1.00	-	1.00	1.00	-	0.58	72.00	70.58-73.42	-
		CC	1.00	0.68-1.46		1.15	0.71-1.87		72.00	68.89-75.11	0.23
	Overdominant	AA-CC	1.00	-	0.95	1.00	-	0.94	72.00	70.19-73.81	-
		AC	0.99	0.72-1.36		0.98	0.64-1.48		72.00	70.12-73.88	0.43
	Log-additive	-	0.99	0.80-1.24	0.96	1.09	0.82-1.44	0.56	-		
rs9821091	Codominant	GG	1.00	-	0.32	1.00	-	**0.045**	72.00	69.77-74.23	-
		GA	0.86	0.61-1.22		**0.81**	**0.52-1.28**		73.00	71.43-74.57	0.11
		AA	1.22	0.76-1.97		**1.75**	**0.95-3.20**		71.00	68.17-73.83	0.16
	Dominant	GG	1.00	-	0.70	1.00	-	0.96	72.00	69.77-74.23	-
		GA-AA	0.94	0.68-1.30		0.99	0.65-1.50		72.00	70.56-73.44	0.42
	Recessive	GG-GA	1.00	-	0.21	1.00	-	**0.02**	72.00	70.76-73.24	-
		AA	1.33	0.86-1.12		**1.95**	**1.12-3.40**		**71.00**	**68.17-73.83**	**0.017**
	Overdominant	GG-AA	1.00	-	0.20	1.00	-	0.085	71.00	69.06-72.94	-
		GA	0.81	0.59-1.12		0.70	0.46-1.05		**73.00**	**71.43-74.57**	**0.018**
	Log-additive	-	1.05	0.83-1.32	0.70	1.19	0.89-1.61	0.24	-		
rs9834412	Codominant	CC	1.00	-	0.94	1.00	-	0.94	73.00	71.02-74.98	-
		CA	0.94	0.67-1.32		1.00	0.65-1.55		72.00	71.10-72.90	0.78
		AA	1.02	0.53-1.95		1.15	0.52-2.55		72.00	64.23-79.77	0.36
	Dominant	CC	1.00	-	0.78	1.00	-	0.91	73.00	71.02-74.98	-
		CA-AA	0.96	0.69-1.32		1.02	0.68-1.55		72.00	71.15-72.86	0.59
	Recessive	CC-CA	1.00	-	0.90	1.00	-	0.72	72.00	70.54-73.46	-
		AA	1.04	0.55-1.97		1.15	0.53-2.50		72.00	64.23-79.77	0.34
	Overdominant	CC-AA	1.00	-	0.73	1.00	-	0.94	72.00	70.22-73.78	-
		CA	0.94	0.68-1.31		0.98	0.64-1.50		72.00	71.10-72.90	0.92
	Log-additive	-	0.98	0.75-1.27	0.86	1.04	0.75-1.44	0.81	-		

OR: odds ratio; aOR: odds ratio adjusted for age and gender; CI: confidence interval.
Values in **bold** are statistically significant (P<0.05).

PS053

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Gene Expression Characterization of the Prostaglandin E_2 (PGE_2) Pathway in Gastric Cancer Development - The Aspirin as an Opportunity for Prevention?

Catarina Lopes[1,2,3], Carina Pereira[1,4], Luís Lima[5], Rui Medeiros[1,6], Mário Dinis-Ribeiro[4,7]

1. Molecular Oncology and Viral Pathology Group, Portuguese Institute of Oncology; 2. Faculty of Sciences of University of Porto (FCUP); 3. Abel Salazar Institute of Biomedical Sciences (ICBAS); 4. CINTESIS - Center for Health Technology and Services Research, University of Porto; 5. Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology; 6. Portuguese League Against Cancer; 7. Gastroenterology Department, Portuguese Institute of Oncology.

1. Introduction

Gastric cancer (GC) is the fifth most common cancer worldwide and the **third leading cause of cancer-related deaths** (1). Prostaglandin E_2 (**PGE_2**) plays important roles in the **development of many types of cancer**, including GC, **contributing to evasion of apoptosis, sustained angiogenesis, and tissue invasion** (2). PGE_2 levels are mainly regulated by cyclooxygenase-2 (**COX-2**), which can be **inhibited** by **nonsteroidal anti-inflammatory drugs** (NSAIDs) such as the **aspirin**, and multidrug resistance protein 4 (**MRP4**), responsible for **PGE_2 synthesis and transport out of the cell**, respectively, and 15-hydroxyprostaglandin dehydrogenase (**15-PGDH**) and prostaglandin transporter (**PGT**), responsible for its **inactivation** (3). Even though there are distinct genetic and molecular signatures across ethnic populations, most published studies are focused on Asian populations.

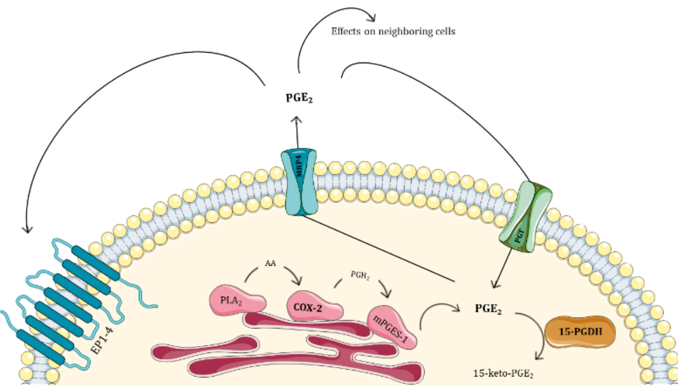
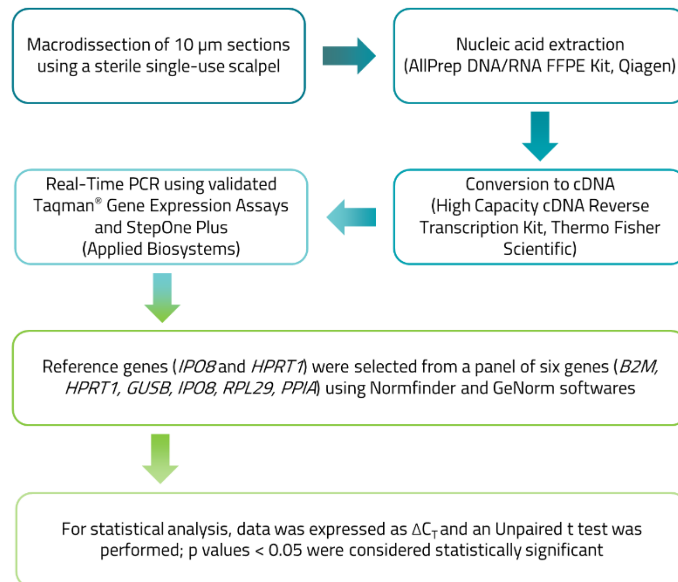


Figure 1. The PGE_2 pathway. The PGE_2 synthetized within the cell is then transported to the extracellular milieu, where it can exert effects in an autocrine or paracrine matter by interacting with the EP1-4 receptors. On the other hand, it can also be transported back into the cell by PGT and be converted to 15-keto-PGE_2, its inactive form, by 15-PGDH.

2. Aims

We aimed to explore the genetic expression of *PTGS2, ABCC4, HPGD,* and *SLCO2A1,* the genes encoding the four proteins mentioned above, in **formalin-fixed, paraffin-embedded (FFPE) samples (tumorous and "normal"-appearing mucosa)** from **Caucasian patients** with histological confirmation of **intestinal-type GC**, consecutively selected after reviewing the histopathological database from the Pathology department at IPO-Porto from January 2013 to December 2015.

3. Material and Methods



4. Results

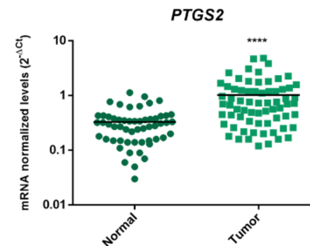


Figure 2. *PTGS2* mRNA expression is upregulated in tumor samples (n=68) compared to samples of "normal"-appearing mucosa (n=56), **by a mean factor of 2.647** (****p<0.0001, Unpaired t test). Lines represent the median values of *PTGS2* gene expression.

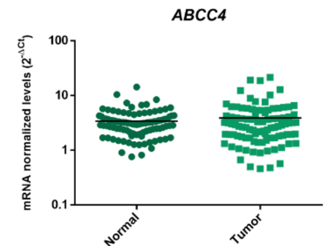


Figure 3. *ABCC4* mRNA expression does not present a statistically significant difference between tumor samples (n=96) and "normal"-appearing mucosa samples (n=99, p=0.2750, Unpaired t test). Lines represent the median values of *ABCC4* gene expression.

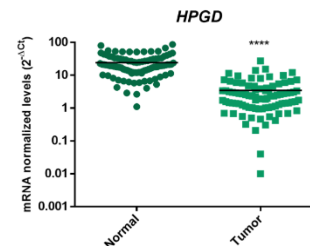


Figure 4. *HPGD* mRNA expression is downregulated in tumor samples (n=90) compared to samples of "normal"-appearing mucosa (n=99), **by a mean factor of 0.101** (****p<0.0001, Unpaired t test). Lines represent the median values of *HPGD* gene expression.

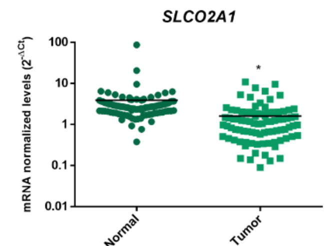


Figure 5. *SLCO2A1* mRNA expression is downregulated in tumor samples (n=87) compared to samples of "normal"-appearing mucosa (n=99), **by a mean factor of 0.359** (*p=0.0184, Unpaired t test). Lines represent the median values of *SLCO2A1* gene expression.

5. Discussion

The role of PGE_2 pathway, mainly of COX-2, has been explored throughout the years in a variety of cancers. However, its role in Caucasian patients with GC remains to be enlightened.

Here, we observed an **upregulation of COX-2** and a **downregulation of 15-PGDH and PGT in tumor samples** compared to "normal"-appearing samples. This pattern is expected to contribute to the **increase of PGE_2 levels in the extracellular environment**, due to the increase of its synthesis and to the decrease of its inactivation. In turn, **that will stimulate well-known hallmarks of cancer** – evasion of apoptosis, self-sufficiency, limitless replicative potential, metastasis – resulting from the interaction between this PG and the prostaglandin receptors (EP1-4) (4).

Our results corroborate the existing reports on this pathway in colorectal, liver, head and neck, pancreas, breast, prostate, esophagus, and bladder cancers (4). On the other hand, no difference was observed regarding MRP4 expression, which has been found to be upregulated in some types of cancer.

Furthermore, if supported by further studies, **aspirin, a known COX-2 inhibitor,** could represent a **targeted chemopreventive strategy for GC in individuals overexpressing COX-2.** This procedure has been shown to be effective in patients with colorectal cancer, decreasing mortality and recurrence (5).

6. References

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