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The impact of aspirin resistance-associated genetic variants in the clinical outcome of epithelial ovarian cancer patients

Márcia Silva Ferreira

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The impact of aspirin resistance-associated genetic variants in the clinical outcome of epithelial ovarian cancer patients

Dissertação de Candidatura ao Grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Abbreviations

A

A	Adenine
AA	Arachidonic acid
ADP	Adenosine diphosphate
AR	Aspirin resistance
ASA	Acetylsalicylic acid

B

BRCA1	BRCA1, DNA repair associated
BRCA2,	BRCA2, DNA repair associated

C

C	Cytosine
CAD	Coronary artery disease
CI	Confidence interval
COX	Cyclooxygenase enzyme
COX-1	Cyclooxygenase enzyme 1
COX-2	Cyclooxygenase enzyme 2
CVD	Cardiovascular disease

D

DC	Dendritic cell
DNA	Deoxyribonucleic acid
DFS	Disease-free survival

E

ECM	Extra cellular matrix
EDTA	Ethylenediamine-tretaacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian cancer
ESC	European Society of Cardiology
EUR	European

F

FDG-β	Fibroblast growth factor beta
FIGO	International Federation of Gynecology and Obstetrics

G

G	Guanine
GP	Glycoprotein
GR	Glutathione reductase
GSR	Glutathione-Disulfide Reductase
GWAS	Genome-wide association study

H

HR	Hazard ratio

I

IARC	International Agency for Research on Cancer
IBS	Iberian
ITGA2	Integrin Subunit Alpha 2
ITGB3	Integrin Subunit Beta 3

L

LD	Linkage disequilibrium
LPA	Lysophosphatidic acid
LTA	Light transmission aggregometry

M

MAF	Minor allele frequency
ME	Microenvironment
MLH1	mutL homolog 1
MSH2	mutS homolog 2
MSH6	mutS homolog 6

N

NC	Non-coding
NSAID	Non-steroidal anti-inflammatory drug
NK	Natural killer

O

OC	Ovarian cancer
OR	Odds ratio
OS	Overall survival

P

PARP	Poly ADP ribose polymerase
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEAR1	Platelet endothelial aggregation receptor
PFA-100	Platelet function analyzer
PG	Prostaglandin
PID	Pelvic inflammatory disease
PMS2	PMS1 homolog 2, mismatch repair system component
PTGS1	Prostaglandin-Endoperoxide Synthase 1
PTGS2	Prostaglandin-Endoperoxide Synthase 2
P2RY	Purinergic Receptor

R

ROS	Reactive oxygen species
RGS7	Regulator of G Protein Signaling 7

S

SNP	Single nucleotide polymorphism

T

T	Thymine
TAMs	Tumor associated macrophages
TBXA2R	Thromboxane A2 receptor
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment

Treg	T-regulatory
TX	Thromboxane
TXA2	Thromboxane A2

V

VEGF	Vascular endothelial growth factor

W

WBA	Whole blood aggregometry
WHO	World Health Organization

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Abstract

Introduction: Over the years, the vital role of inflammation in oncology became increasingly evident, specifically concerning the chronic inflammation, being even recognized as a cancer hallmark since 2011. This physiological condition appears to influence the development and progression of numerous types of cancer, namely ovarian cancer (OC) due to the repetitive inflammatory states registered in ovaries throughout a women's reproductive life. In this sense, it is considered that the anti-inflammatory drugs' consumption, as aspirin, exhibit a protective effect in oncologic diseases. Nevertheless, there is a considerable proportion of patients who do not benefit from aspirin anti-inflammatory effect, in a phenomenon known as aspirin resistance (AR). Whilst AR etiology is likely to be multifactorial, genetic factors appear to be preponderant. According to several genetic association studies, as genome-wide and candidate gene studies, numerous single nucleotide polymorphisms (SNPs) have been identified mostly in cyclooxygenase, thromboxane, and platelet receptors-related genes as capable to negatively affect the aspirin action. However, it remains unclear the role of AR-associated genetic variants in oncology, specifically regarding OC patients. Thereby, the purpose of the present study was to evaluate the influence of six selected AR-variants in the clinical outcome of a cohort of epithelial ovarian cancer patients (EOC) from the North region of Portugal.

Methods: After the compilation of all AR-identified variants, specific selection criteria were applied, based on priority ranking generate by GWAS4D, minor allele frequency (MAF) in the Iberian population (>10%), availability of the respective validated genotyping assay and, ultimately, the putative relevance in AR and cancer biological pathways, in order to select the most suitable variants to be studied. A retrospective hospital-based cohort study was performed, including 336 EOC patients submitted to first-line treatment. Polymorphism genotyping was conducted using TaqMan® Allelic Discrimination methodology through the Real-Time Polymerase Chain Reaction (PCR) technique. Overall survival (OS) and disease-free survival (DFS) at 5-years were the two clinical endpoints established in this study. All tests were two-sided, and a level of $P<0.05$ was considered as statistically significant.

Results: Taking into account the considered criteria, the six most prioritized AR-related SNPs were selected, namely *PTGS2* rs20417, *ITGB3* rs5918, *TBXA2R* rs1131882, *PEAR1* rs12041331, *RGS7 rs2502448,* and *GSR* rs3779647. Among them, four genetic variants showed to be significantly associated with the clinical outcome of EOC patients. Particularly in the early stages, patients carrying *ITGB3* rs5918 TT genotype had a reduced survival time when compared with C-allele carrier patients (log-rank test, $P=0.036$). In addition, for patients with FIGO I/II stage at diagnosis who were submitted to incomplete/sub-optimal cytoreduction, a significantly improved survival and a prolonged time until disease

recurrence was observed among *TBX2AR* rs1131882 GG genotype patients when compared to heterozygous patients (log-rank test, *P*=0.029 and *P*=0.002, respectively). For the same subgroup, a significantly decreased survival time was observed in patients carrying *RGS7* rs2502448 TT genotype when compared to the C allele carriers (*P*=0.035). Nevertheless, none of these associations retain statistical significance in the multivariate analyses (*P*>0.05). For the subgroup of patients with advanced disease stage at diagnosis and with more than 1 cm of residual disease, *GSR* rs3779647 C allele carriers showed a significantly diminished survival time compared with the reference genotype (TT) carriers (log-rank test, *P*=0.010). In accordance, *GSR* rs3779647 polymorphism emerged as one of the most relevant predictors of EOC death risk at 5-years, being a potential biomarker to evaluate clinical outcome in this clinical setting. In fact, this variant appears to be involved with a higher insensitivity to platinum-based first-line chemotherapy, likely due to its role in glutathione metabolism and, consequently, with the detoxification of this cytotoxic compound.

Conclusion: Although this study did not provide definitive conclusions regarding the relevance of AR-genetic variants in the prognosis of EOC patients, it suggests that some of these SNPs might have a prognostic value in this clinical setting. Therefore, further functional/fine-mapping analyses are indispensable to understand the biological mechanisms underlying the achieved results and hence, to attest the clinical relevance of studied polymorphisms for EOC patients.

The trend in literature appears to confirm the importance of aspirin as OC adjuvant therapy and future clinical studies might have in consideration the influence of the genetic background to optimize treatment strategies, in the scope of personalized medicine.

Keywords: Aspirin; aspirin resistance; single nucleotide polymorphisms; genetic association studies; GWAS; candidate gene studies; epithelial ovarian cancer; clinical outcome

Resumo

Introdução: Ao longo dos anos, tornou-se evidente o papel essencial da inflamação no cancro, especialmente da inflamação crónica, sendo desde 2011 reconhecida como um *hallmark* do cancro. Esta condição fisiológica parece influenciar o desenvolvimento e progressão de vários tipos de cancro, nomeadamente o cancro do ovário (CO), dado o repetido estado inflamatório observado nos ovários ao longo da vida reprodutiva da mulher. Neste sentido, considera-se que o consumo de agentes anti-inflamatórios, como a aspirina, possa ter um efeito protetor nas doenças oncológicas. No entanto, existe uma proporção considerável de indivíduos que não beneficia do efeito anti-inflamatório da aspirina, num fenómeno designado como resistência à aspirina (AR). Apesar da etiologia da AR ser provavelmente multifatorial, os fatores genéticos parecem ter um papel preponderante no seu desenvolvimento. De acordo com vários estudos de associação genética, nomeadamente estudos gene candidato e *genome-wide,* vários polimorfismos de nucleótido único (SNPs) têm sido identificados, sobretudo em genes associados à ciclooxigenase, tromboxano e a recetores plaquetários, como capazes de afetar negativamente a ação da aspirina. Contudo, permanece por esclarecer o papel das variantes genéticas associadas à manifestação de AR em oncologia, nomeadamente em doentes com CO. Deste modo, o presente estudo tem como objetivo avaliar a influência de seis polimorfismos associados a AR na evolução clínica de uma coorte de doentes com cancro epitelial do ovário (CEO) da região Norte de Portugal.

Métodos: Após a compilação de todas as variantes genéticas associadas a AR, foram aplicados critérios específicos baseados na priorização estabelecida através do software GWAS4D, na frequência do alelo menos comum na população Ibérica (>10%), na disponibilidade dos respetivos *assays* de genotipagem, na potencial relevância nas vias biológicas envolvidas na AR e em oncologia, de modo a selecionar as mais adequadas a implementar no presente estudo. Consequentemente, foi desenvolvido um estudo do tipo coorte retrospetivo de base hospitalar, envolvendo 336 pacientes com CEO submetidas a tratamento de primeira linha. A genotipagem dos polimorfismos foi realizada recorrendo à metodologia de discriminação alélica TaqMan®, através da técnica de reação em cadeia da polimerase em tempo real (PCR). A evolução clínica foi avaliada neste estudo recorrendo à análise da sobrevivência global (SG) e da sobrevivência livre de doença (SLD) a 5 anos. Todos os testes estatísticos foram bilaterais e o nível de significância estabelecido foi de 5%.

Resultados: Atendendo aos critérios considerados, selecionamos seis variantes de elevada priorização associadas com AR, nomeadamente *PTGS2* rs20417, *ITGB3* rs5918, *TBXA2R* rs1131882, *PEAR1* rs12041331, *RGS7 rs2502448* e *GSR* rs3779647. Entre estas, quatro variantes demonstraram estar estatisticamente associadas com a evolução

clínica das doentes com CEO. Particularmente em estadios precoces, as doentes portadoras do genótipo *ITGB3* rs5918 TT apresentaram um menor tempo de sobrevivência a 5 anos quando comparado com portadoras do alelo C (teste *log-rank*, *P*=0.036). Além disso, para as doentes diagnosticadas em estadios iniciais e que foram submetidas a cirurgia citorredutora incompleta/subótima, foi observada uma melhoria significativa tanto na SG quanto na SLD a 5 anos entre as portadoras do genótipo *TBX2AR* rs1131882 GG, em comparação com portadoras do genótipo AG (teste *log-rank*, *P*=0.029 e *P*=0.002, respetivamente). Ainda neste subgrupo, observamos uma diminuição no tempo de sobrevivência entre as portadoras do genótipo *RGS7* rs2502448 TT quando comparado com o alelo C (*P*=0.035). No entanto, nenhuma associação estatisticamente significativa foi reforçada na análise multivariada (*P*>0.05). Dentro do subgrupo de doentes diagnosticadas em estadios avançados e com doença residual superior a 1 cm, as portadoras do alelo *GSR* rs3779647 C demonstraram um menor tempo de sobrevivência quando comparado com as portadoras do genótipo de referência (TT) (teste *log-rank*, *P*=0.010). Neste sentido, o polimorfismo *GSR* rs3779647 surge como um dos preditores mais relevantes do risco de morte a 5 anos por CEO, sendo um potencial biomarcador para avaliação da evolução clínica destas doentes. De facto, este polimorfismo parece estar envolvido com uma maior insensibilidade ao tratamento de primeira linha, nomeadamente quimioterapia baseada em platinos, possivelmente devido ao seu papel no metabolismo da glutationa e, consequentemente, na destoxificação deste agente citotóxico.

Conclusão: Apesar de este estudo não possibilitar conclusões definitivas sobre a relevância dos polimorfismos associados a AR no prognóstico das doentes com COE, algumas destas variantes possam apresentar um valor prognóstico neste grupo clínico. Consequentemente, futuras análises funcionais e de mapeamento são indispensáveis para compreender os mecanismos biológicos subjacentes aos resultados apresentados e, assim, comprovar a relevância clínica dos polimorfismos estudados para doentes com COE.

As evidências na literatura parecem confirmar a importância da aspirina como terapia adjuvante no CO e, como tal, estudos clínicos futuros devem ter em consideração a influência do perfil genético para a otimização das estratégias terapêuticas, no âmbito da medicina personalizada.

Palavras-chave: Aspirina; resistência à aspirina; polimorfismos de nucleótido único; estudos de associação genética; GWAS; estudos gene candidato; cancro do ovário epitelial; evolução clínica

1. Introduction

Chapter 1: Oncobiology and molecular epidemiology

Despite the improvement in screening, early diagnosis and treatment over the years, cancer continues to be one of the leading causes of death worldwide, representing a global and growing concern to the medical community [1-3]. In 2018, the International Agency for Research on Cancer (IARC) estimated that about 18.1 million of new cases and 9.6 million deaths were due to this pathology, being the lung and breast cancers the most common and lethal malignancies in man and woman, respectively (18.4% and 6.6% of total cancer deaths). The expansion of population and its aging, as well as the alterations in prevalence and distribution of risk factors related to cancer susceptibility, have been pointed as the main reasons for the increase of cancer incidence [4]. Thus, in 2030 the number of new cancer cases is expected to increase to around 22.2 million [5].

Cancer is considered a hyperproliferative disorder that involves transformation of cell morphology, dysregulation of the apoptotic process as well as uncontrolled cell proliferation/invasion, angiogenesis and dissemination [6, 7]. However, at the genomic level, cancer is characterized by an ample range of dynamic changes that lead to the deregulation of biological routes and processes, affecting several cellular systems, ranging from molecular activity to cellular communication [8]. Overall, carcinogenesis is considered as a three-stage process, involving initiation, promotion, and progression [9]. The initiation phase is considered to lead to the acquisition of non-lethal permanent genomic damage by normal cells, caused through the action of physical, chemical and biological carcinogenic agents, epigenetic changes or through the inheritance of genetic alterations in the germline, becoming then susceptible to malignant transformation/development. Whether the initiated cell be conveniently stimulated by promotor agents, it might occur the progressive accumulation of genetic changes that provide a selective advantage for the initiated cell when compared with the remaining normal cells. Thus, promotion is characterized by the selective clonal expansion of initiated cells and, despite being considered as a reversible process, the constant promotion of cell proliferation enhances the damage propagation caused by initiation which, in turn, increase the risk of additional mutations acquisition and the establishment of a malignancy state [9-11]. The expression of a malignant phenotype by the accumulation of additional mutations in propagated cells is commonly observed in the progression stage [10].

The carcinogenic process culminates with modifications in tumor cell physiology which are responsible for the acquisition of specific hallmarks, such as: evasion to apoptosis, insensitivity to inhibitory signals of growth and self-sufficiency to these signals, unrestrictive replicative potential, modified cellular metabolism, sustained angiogenesis, tissue invasion and metastasis and, ultimately, the ability to escape to immune surveillance

[6, 12]. Furthermore, genetic instability and cancer-associated inflammation are additional factors that have also proved to be crucial and transverse to the most tumors (Figure 1) [12]. However, a tumor should not be stated as an inert mass of cells but rather as a dynamic process where the interactions with the microenvironment should be considered since contribute with external signals for the development and manifestation of the malignant phenotype [12, 13].

Given its peculiarities and significant impact on public health, cancer has been subject of intensive study over the years, although several issues remain unclear.

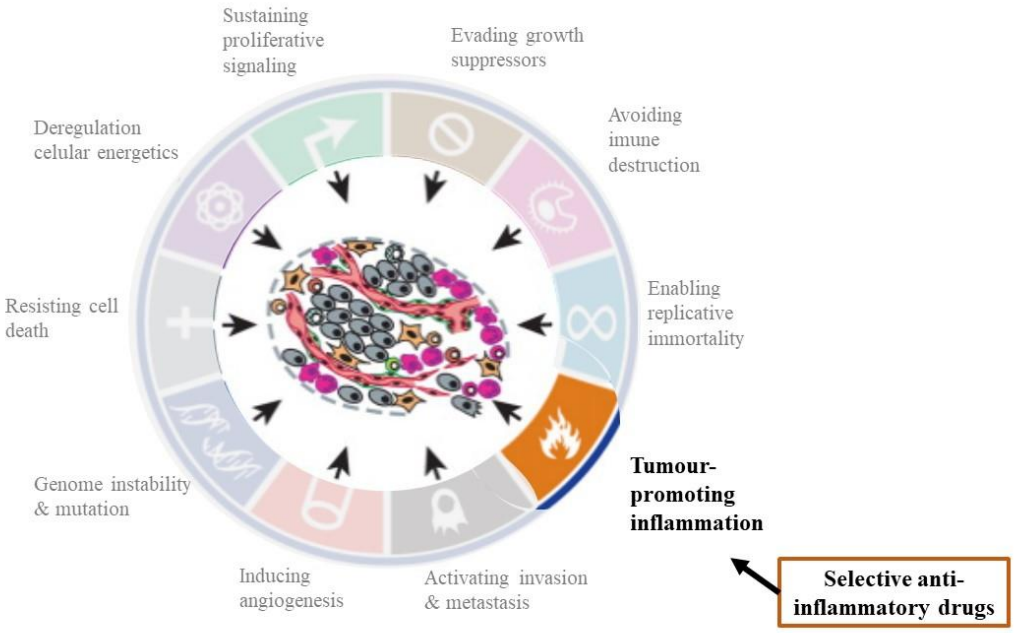

Figure 1 - The Hallmarks of cancer: focus on tumor-promoting inflammation and its therapeutic target (adapted from [12]).

Chapter 2: The crucial role of inflammation in cancer

The functional relationship between cancer and inflammation is old, being firstly described in the 19th century by Rudolf Virchow [14]. This link has started based on the observation of biopsied samples from malignant neoplasms with presence of inflammatory cells and on the development of tumors in regions with a high inflammatory burden [15]. Over the years, the crucial role of inflammation in cancer became increasingly evident, being recognized as a cancer hallmark since 2011. In fact, inflammation is considered to contribute to the acquisition of additional characteristics by tumor cells, since a pro-inflammatory state supplies the tumor microenvironment, as described below [12].

Overall, inflammation is a physiologic reaction prompt by the organism in response to several processes, such as infection, injury or irritation, whose ultimate goal is to restore

tissue homeostasis through the destruction or healing of the damaged tissue [16, 17]. In this sense, regarding clinical manifestation, the inflammatory process is characterized as following five cardinal signs, namely redness, swelling, heat, pain, and loss of function [18].

The inflammatory process can assume either an acute or a chronic manifestation. Contrary to acute inflammation, which is triggered for a short period of time and usually in benefit of the host, chronic inflammation response persists for a longer period and may predispose the subject to several diseases, like carcinogenesis [7]. In fact, epidemiological data showed that over 25% of all cancers are associated with infectious diseases and chronic inflammation [19, 20]. Chronic inflammation contributes to tumor development through several mechanisms, including the induction of DNA damage and genomic instability in part due to the production of reactive oxygen species (ROS), which may lead to genetic and epigenetic modifications, ultimately triggering cellular transformation. Furthermore, inflammation might also influence tumor progression by supporting angiogenesis, inducing tissue remodeling and the production of pro-oncogenic growth factors and cytokines [21, 22].

Tumors considered to be related to chronic inflammation states may be encompassed into two distinct pathways: an extrinsic pathway, conducted by inflammatory conditions that increase tumor susceptibility (such as in the case of inflammatory bowel disease and colon cancer); and, in contrast, an intrinsic pathway, conducted by genetic changes, namely in oncogenes, that trigger tumor development and the establishment of an inflammatory microenvironment without an underlying inflammatory state [14, 23]. These two pathways might converge, leading to transcription factors activation and the production of inflammatory mediators, such as cytokines, chemokines, prostaglandins, among others, that are relevant in carcinogenesis [24].

In summary, chronic inflammation appears to be involved in different stages of carcinogenesis, from initiation and promotion to progression, being a putative key factor in the etiology of various types of cancer (Table 1) [7]. Namely, due to repetitive inflammatory states in the ovarian tissues throughout a women's reproductive life, it is highly recognized that the development and progression of ovarian tumors might be closely related to this physiological condition.

Table 1 - Cancers considered to be related to chronic inflammation.

Cancer	Inflammatory conditions
Colorectal cancer	Inflammatory bowel disease, Crohn's disease, chronic ulcerative colitis
Lung carcinoma and mesothelioma	Chronic bronchitis, silicosis, asbestosis
Esophageal cancer	Gastroesophageal reflux and Barret's esophagus
Gastric cancer	Gastritis, ulcers
Pancreatic cancer	Pancreatitis
Gall Bladder cancer	Chronic cholecystitis
Ovarian cancer	**Pelvic inflammatory disease, endometriosis and polycystic ovarian syndrome**
Postate cancer	Chronic prostatitis
Bladder cancer	Chroic cystitis, bladder inflammation
Papillary thyroid cancer	Thyroiditis
Oral squamous cell carcinoma	Gingivitis
Hepatocellular carcinoma	Hepatitis
Bone cancer	Chronic osteomyelitis
Malt lymphoma	Gastritis, ulcers, Sjogren's syndrome, Hashimoto's thyroiditis

2.1. Inflammation and cancer: focusing on ovarian cancer

As the etiology and the initial events of ovarian carcinogenesis are not entirely established, several hypotheses have been proposed which, based on physiological and epidemiological data, pretend to explain, though incompletely, the origin of this neoplasia. Specifically, it has been shown that ovulation itself may have a pro-inflammatory and mutagenic character, as postulated by the incessant ovulation hypothesis [25, 26]. There is growing evidence suggesting that both ovarian epithelium and fallopian tube are continuously exposed to an inflammatory environment caused by common processes related to ovulation (Table 2) [27]. Particularly, the continuous rupture, remodeling and repair mechanisms inherent to the ovulatory cycles increase the concentration of inflammatory mediators, namely cytokines, growth factors, ROS, prostaglandins, and eicosanoids, that promote an oxidative stress state [28, 29]. The repeated secretion of these inflammatory mediators throughout the woman's lifetime generates a chronic inflammatory microenvironment (ME) in the peritoneum which contributes to the uncontrolled cell proliferation, malignant transformation and cell survival within ovarian tissues. Besides, it is considered that the pro-inflammatory ovarian tumor microenvironment (TME) may

potentiate the establishment of ovarian cancer (OC) metastasis and, inclusively, chemoresistance phenotypes [17]. Specifically, a pro-inflammatory ME may contribute to genetic instability and, as the continuous action of inflammatory mediators may lead to DNA damage in epithelial ovarian and fallopian tube cells, influencing apoptotic pathways and initiating the transformation of normal cell. In addition, whether cells transformed either by oncogenic changes or by inflammation exposure are in an inflammatory ME, they may activate the pro-survival signaling pathways rather than the senescence pathways, frequently induced in normal cells. On the other hand, transformed epithelial cells might promote a pro-inflammatory environment through the secretion of inflammatory mediators which may reprogram the adjacent cells to create a profitable TME and hence, promote OC development [12, 17, 30, 31].

Furthermore, OC cells could also produce specific inflammatory mediators that may: 1- lead to immune cells recruitment into the TME (such as Dendritic cells (DC), Tumor-associated macrophages (TAMs), Natural killer (NK) and T-regulatory (Treg) cells), which, in turn, induce the production of additional inflammatory mediators that further prompt the chronic inflammation; 2- stimulate the tumor cells, TAMs and the local fibroblasts to proliferate and produce growth factors (as TGF-β and FGF), that increase the integrins/matrix metalloproteins production, leading to tumor cell migration through degradation of the extracellular matrix (ECM); 3- induce endothelial cells to secrete growth factors such as PDGF, EGF, and VEGF that promote angiogenesis. Consequently, it is considered that OC cells might act as inflammatory enhancers and be induced by inflammatory mediators, as described below (Figure 2) [17, 31, 32].

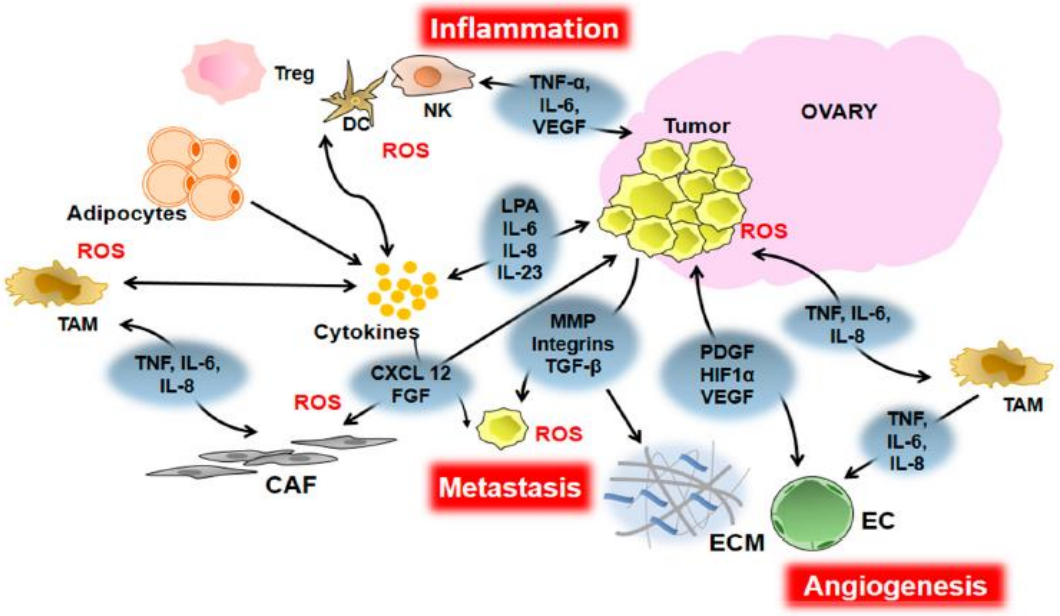

Figure 2 - The role of inflammatory mediators to OC progression, highlighting the impact on metastasis and angiogenesis [17].

Despite inflammation be a key factor that links the several theories proposed to OC carcinogenesis, namely the incessant ovulation, gonadotropins and hormonal, the emergence of inflammation as an individual hypothesis was mainly due to the inability of the remaining theories to adequately explain the increased risk of OC associated with specific inflammatory conditions. Due to the physiological role of inflammation in the ovary, it has been implicated in several ovarian pathologies, such as pelvic inflammatory disease (PID), endometriosis and polycystic ovarian syndrome (PCOS) [28, 33-35]. Additionally, obesity and talc exposure are other sources of ovarian inflammation that appear to have a role in the malignant development [36, 37].

In summary, ovaries exhibit a pro-inflammatory network since its primary physiological function rely on monthly ovulation, an event closely associated with inflammatory processes. Chronic inflammation induces the secretion of several inflammatory mediators, able to potentiate the ovarian cells malignant transformation and, hence, OC initiation. On the other hand, the transformed cells can produce additional inflammatory mediators that promote a continuing inflammatory state. This inflammatory ME may aid premalignant cells in the avoidance of apoptosis, the escape to the immune surveillance and to the uncontrolled growth as well as to potentiate migration/invasion of tumor cells and angiogenesis, which facilitate OC progression and metastasis [17, 27, 38]. For these reasons, inflammation might represent a susceptibility and prognosis factor for OC patients [39]. In addition, several lines of evidence show that the consumption of anti-inflammatory drugs, as the case of aspirin, correlates with the OC susceptibility and clinical outcome in this clinical setting [17].

Table 2 - Evidences that link inflammation to ovarian cancer.

○ Pro-inflammatory mediators (mostly cytokines) are markedly elevated in OC
○ Pro-inflammatory mediators are markedly elevated in diseases states, namely endometriosis and PID, pathologies considered to be associated with an increased risk to ovarian malignancies development.
○ Elevated serum levels of C-reactive protein, used as inflammatory marker, has been correlated with a subsequent increased risk of OC.
○ Ovarian tumor tissues appear to exhibit inflammatory signals such as: redness, swelling and loss of function.
○ Oral contraceptive pills, associated with the reduced the risk of OC, confer a number of biological effects that may mitigate the inflammatory influence, which include ovulation inhibition, PID risk reduction and endometriosis reversal.
○ COX-2 overexpression (activated under inflammatory conditions) was found in the EOC cells, being particularly associated with a poor prognosis.
○ The literature showed the protective effect of anti-inflammatory drugs, namely aspirin, in OC development and progression.

Abbreviations: OC ovarian cancer; PID, pelvic inflammatory disease

Chapter 3: Aspirin and the emergent phenomenon of aspirin resistance

Acetylsalicylic acid (ASA), commonly known as aspirin, has become one of the most widely used and effective non-steroidal anti-inflammatory drugs (NSAID), mainly due to its anti-inflammatory, analgesic and anti-pyretic properties [40]. Briefly, ASA acts through the irreversible inhibition of the cyclooxygenase enzyme (COX), an enzyme involved in the biosynthesis of prostaglandins (PG), which are essential components of the inflammatory process [41, 42]. In humans, two isoforms of the COX enzyme can be expressed, COX-1 and COX-2. Under physiological conditions, COX-1 is constitutively expressed in most tissues, catalyzing the conversion of arachidonic acid (AA) into the prostaglandins G2 and H2 (PGG2 and PGH2, respectively). Subsequently, through the action of thromboxane synthase, those PGs are converted into thromboxane A2 (TXA2), which is a vasoconstrictor and a potent activator of platelet aggregation [43]. In contrast, COX-2 expression is exclusively induced under inflammatory stimuli, leading to the conversion of AA into several distinct PGs, such as PGI2 and PGE2, which are relevant mediators of inflammatory, fever and pain-associated mechanisms [44].

As the mechanism of action, aspirin leads to the acetylation of the serine residues at positions 529 and 516 of COX-1 and COX-2 proteins, respectively, leading to their enzymatic inhibition and, consequently, hampering the above described processes [45-47]. Ultimately, due to the non-conversion of the AA into TXA2, platelet aggregation is suppressed. As secondary effects of platelet aggregation inhibition, there is an attenuation in the ROS, pro-inflammatory cytokines and growth factors release, which reduce the inflammatory process and, thus, leading to an improvement in the endothelial function [42, 48]. As the affinity of aspirin is considered to be highly superior for COX-1 than for COX-2, low ASA therapeutic doses (75-300mg, depending on the administration route) solely inhibit COX-1 being, therefore, associated with an antiaggregant effect [49].

For this reason, this drug is commonly used in clinical practice. As it is well-known, aspirin is prescribed not only for treatment but also for the prevention of atherothrombotic events. Specifically, aspirin might be used to prevent the first occurrence of cardiovascular diseases (CVDs) (primary prevention). With a role in the secondary prevention, long-term aspirin administration has been shown to significantly reduce the risk of non-fatal stroke and acute myocardial infarction as well as death due to vascular causes in high-risk individuals (i.e., those with a personal history of CVD) [11, 12]. Over the last years, the profitable role of aspirin became evident for secondary prevention of CVD, which outbalance the risks associated with its administration, namely for the increased risk of bleeding events occurrence [9]. Nevertheless, due to the lack of robust evidence, the use

of aspirin for the CVD primary prevention setting is not yet consensual, although the extensive debate in the field [13].

In addition to the established relevance for CVDs, ASA has demonstrated an important role in oncology [14]. In fact, CVDs and malignant tumors are among the leading causes of mortality (first and second cause, respectively) and morbidity worldwide, which represent a major concern for the medical community. Therefore, the definition and application of preventive strategies to mitigate the impact of these pathologies is essential which might benefit, for instance, from the aspirin administration [15, 16].

The chemopreventive effect of aspirin was firstly described for colorectal cancer patients, although its beneficial effect is thought to be transversal to several other tumors, including OC [50-55]. The anti-tumoral effect of ASA appears to be proportional to the consumption duration, being more evident in cases of long-term daily aspirin consumption (e.g., at least five years). However, no definitive conclusions were achieved regarding the optimal dose required to produce the greater and an effective anti-tumoral effect [50, 52, 56, 57]. Additionally, ASA is considered to reduce cancer morbidity and mortality which suggests that this antiplatelet drug not only prevents the development of malignancies but also has an influence on cancer prognosis[50]. Despite the mechanisms underlying the protective effect of ASA in cancer development and progression are not entirely established yet, it is proposed that they might be driven by the direct inhibition of COX enzymes, particularly COX-2, closely related to the inflammatory process [58]. In fact, the literature describes several pro-tumoral effects of COX-2, which include the stimulation of angiogenesis, resistance to apoptosis, increased invasiveness and DNA mutagenesis [59, 60]. Furthermore, COX-2 might stimulate the aromatase enzyme expression and, in turn, increase the conversion of androgen to estrogen, a recognizer promoter of tumor growth [61]. For instance, the putative involvement in hormonal metabolism is taken into account to explain the beneficial/preventive effect of ASA, particularly in ovarian and other hormone-related tumors. However, the anti-cancer effect of low doses of ASA might not be explained by these COX-2 mediated mechanisms. To clarify this issue, some theories have been proposed, namely that the inhibition of COX-1 by aspirin leads afterwards to the suppression of COX-2 expression and to the subsequent downstream signaling in adjacent cell types [62, 63]. Besides, the antiplatelet effect of aspirin caused by COX-inhibition may diminish the direct interaction of platelets with cancer cells and, thus, prevent the development of metastasis and the establishment of a metastatic niche [58]. Nevertheless, further investigation is needed to clarify the relevance of ASA administration in this clinical setting [64].

3.1. Aspirin resistance

Despite the benefits associated with aspirin, it is estimated that 2-57% of ASA users exhibit a suboptimal response to this compound. Consequently, a proportion of individuals does not respond to the action of this drug and may suffer recurrent thromboembolic vascular events, in a process known as aspirin resistance (AR) [65, 66]. The AR remains poorly defined and no consensual characterization has been established yet, leading to inconsistent reports of AR in the literature [66]. To overcome this issue, the European Society of Cardiology (ESC) Working Group on Thrombosis subclassified AR according to two distinct features: clinical and laboratory resistance. Thus, the failure to prevent cardiovascular events in patients treated with ASA is recognized as clinical AR (or clinical treatment failure) [67]. In agreement, individuals encompassed within this AR subcategory can only be identified retrospectively, as the thrombotic events must occur after aspirin treatment initiation [68]. It is estimated that about 8-18% of aspirin-treated individuals will eventually experience clinical treatment failure after two years of therapy initiation [69].

In contrast, laboratory AR is considered when *in vitro* platelet reactivity is not properly blocked, even with the administration of ASA. This occurs in the case of improper inhibition of TXA2 synthesis or platelet aggregation-related to TXA2 production [67]. Laboratory AR is determined by a variety of laboratory assays that assess platelet function or quantifies metabolites levels (as described in Table 3). Nevertheless, despite the availability of several laboratory methods, none of them is considered to be highly specific or consistent over time [65, 70, 71]. Additionally, despite these assays provide relevant information regarding the biochemical and functional features of AR, individually they are not considered to be suitable to guide putative therapeutic decisions [67].

Table 3 - Comparison between the several methods used to evaluate platelet function and, thus, laboratory aspirin-resistance.

Procedures	Principle of action	Advantages	Limitations
LTA	Measures the optical density through a platelet-rich plasma suspension in response to classic agonists (AA, ADP)	- The most specific method to evaluate the response to aspirin (considered as the gold standard) - Widely available - Correlated with clinical events	- Requires intensive labour - Highly dependent on sample preparation - Poorly reproducible
WBA	Monitor of changes in impedance in response to classic agonists	- Consider the interactions between platelets with the other blood cellular constituents Less sample preparation requirements	- Does not overcome all the LTA's limitations.
VerifyNow ®	Fully automated platelet aggregometer to assess antiplatelet therapy	- Well correlated with optical aggregometry (general agreement) - Low blood sample required - Correlated with clinical events	-Nonflexible - Expensive - Uncertain reproducibility
PFA-100 ®	High-shear platelet adhesion and aggregation during the formation of a platelet plug	- Simple, fast test - Low blood sample required - Good reproducibility/sensitivity - Correlated with clinical events	- Not an aspirin-specific test; - Hematocrit and plasma von-Willebrand factor-dependent - Poorly reproducible
Determination of plasmatic TxB2 level	Evaluates the level of the main metabolite of TxA2, after aggregation of platelet rich-plasma	- Low blood sample required	- Possibility of artifacts - Nonlinear correlation with TxA2-induced platelet aggregation
Determination of 11-dehydro-TxB2 in urine	Evaluates the level of the main metabolite of TxA2 in urine	- Non-invasive technique - Total activity of TxA2 determined	- Non-platelet sources of TxA2 are distinguished by this technique
Flow cytometry	Fluorescent determination of platelet activation markers (e.g, P.selectin,) and conformational alterations in platelet glycoproteins	- High Sensitivity (without the need of agonist) - Quantitative test (high specificity) - Low blood sample required	- Expensive - Need for highly specialized laboratory centers
Impact-R ®	Monitor platelet adhesion to polystyrene surface coated with blood plasma proteins	- Low blood sample required	- Weak correlation with other techniques

Abbreviations: AA, acid arachidonic; ADP, Adenosine diphosphate; COX, cyclooxygenase; LTA, light transmission aggregometry; PFA-100, platelet function analyzer; TXB2, thromboxane B2; WBA, whole blood aggregometry

The concept of AR can also be considered according to pharmacokinetic and pharmacodynamic properties. In the case of pharmacokinetic resistance, the limited bioavailability of the active drug at the level of its target is the primary reason for low biological response to aspirin. In this case, the *in vitro* addition of aspirin to a blood sample may block or significantly reduce platelet aggregation and, hence, TXA2 concentration. In contrast, pharmacodynamic resistance may occur due to changes in COX-1 expression, the main aspirin target enzyme, and so the addition of aspirin *in vitro* to blood samples would not alter the production of TXA2 significantly [65, 72, 73]. Nonetheless, based on ESC Working Group guidelines, laboratory resistance should only be considered when alterations in the ASA target enzyme impair the expected effects conferred by this drug [72]. Finally, another subgroup of AR has also been proposed, named pseudo-resistance, in which TXA2 is efficiently suppressed although the platelet activation occurs [74]. Despite providing a helpful tool to study the putative biological mechanisms of AR, pharmacokinetic and pharmacodynamic models have not yet reached clinical utility [75].

Thereby, considering the difficulties and controversies encompassing the AR concept, specific AR diagnosis criteria have not yet been systematically implemented in the clinical practice [71]. However, the criterion proposed by Gum *et al*. (2001) is the most frequently used and recognized by researchers. Namely, it is defined as the mean aggregation of above 70% with 10 µmol/mL adenosine diphosphate (ADP) and a mean aggregation of above 20% with 0.5 mg/mL of AA [76].

3.1.1. Possible causes of aspirin resistance and/or non-response

Although the precise mechanisms underlying AR are not yet well established, AR etiology is believed to be multifactorial and, therefore, distinct hypotheses have been proposed regarding the individual non-response to aspirin [68]. There are several reasons why ASA may not suppress TXA2 expression and impair platelet aggregation inhibition, both considering non-genetic and genetic causes [41, 75].

3.1.1.1. Non-Genetic causes

Poor Adherence

The lack of adherence to therapy is a factor commonly neglected, explaining, perhaps, the ineffectiveness of aspirin in the clinical and laboratory scope [75]. There is a considerable number of patients who do not use aspirin consistently, being improperly identified as AR individuals [41]. It is estimated that around 40% of patients with cardiovascular complications do not comply with aspirin treatment schemes, being the

aspirin-adverse effects, such as bleeding, one of the main barriers to its intake [77-79]. Thus, distinguish between aspirin non-responders and poor ASA-therapy adherence is crucial.

Dosage

The association between aspirin dosage and AR is controversial. According to a prospective study performed by Kojuri and colleagues (2010) about the effect of ASA on platelet function, the manifestation of AR is not dose-dependent [80]. Inclusively, it was suggested that high-aspirin dosage does not confer a significant great efficacy compared to the recommended low doses (75-150mg/day), but instead increase the risk of adverse cardiovascular events [81, 82]. However, some studies reported that increasing aspirin dosages would decrease AR prevalence [83-85].

Drug interactions

The interaction between concomitant medication may be another explanation for the development of AR, especially considering pharmacokinetic resistance. A considerable percentage of AR individuals were found among those who would also take additional medications, like statins, other NSAIDs and proton pump inhibitors [86, 87]. Specifically, the concomitant use of other NSAID-class drugs may decrease the antiaggregant effect of aspirin, through the competition with the COX-1 enzyme binding site [49]. For instance, Ibuprofen is a commonly prescribed medication known to interact with the action of aspirin, contrasting with diclofenac and rofecoxib (a selective COX-2 inhibitor) which do not show any influence [87]. Furthermore, the intake of proton pump inhibitors has been reported to decrease the bioavailability of aspirin due to the increased activity of gastrointestinal mucosa esterases, involved in ASA hydrolyzation, leading to its reduced absorption [48].

Platelet turnover

Another postulated AR-contributing mechanism is platelet turnover. Considering the short aspirin half-life and the high platelet turnover characteristic of clinical conditions, such as inflammation, infection or post-operative periods, an increase in the proportion of platelets not targeted by ASA may be noted. Thereby, this condition may promote an impaired suppression of platelet COX-1, reducing then the effectiveness of aspirin treatment [88].

Tachyphylaxis

Prolonged administration of aspirin may reduce its antiplatelet effect, a condition known as tachyphylaxis. The mechanisms inherent to this process remain quite unknown, although it seems that the clinical effectiveness of aspirin regarding CVDs decreases progressively during long-term treatment, with an increased incidence of adverse

cardiovascular outcomes in these patients [89]. Nevertheless, one of the possible explanations to tachyphylaxis is the progressive reduction of adherence to therapy over time [75, 90].

Other sources of thromboxane production and platelet activation

Other pathways are not blocked by ASA but may also be responsible for thromboxane biosynthesis and, therefore, with a role in AR establishment, namely platelet COX-2, platelet COX-independent thromboxane production or non-platelet thromboxane production pathway. Regarding the latter, rather than being expressed in platelets, TXA2 is produced by nucleated blood cells, as monocytes and macrophages, cell types that are often insensitive to the action of aspirin [41, 91, 92].

Additionally, alternative pathways responsible by platelet activation may not be inhibited by aspirin, like those encompassing non-TXA2-dependent activators as collagen, ADP thrombin and epinephrine [69, 93].

Medical history/Personal lifestyle

In addition to the previously mentioned factors associated with AR, clinical conditions as obesity, diabetes, hypertension and hyperlipidemia as well as smoking habits should be taken into consideration to stratify the individuals according to AR-risk groups since they may influence the therapeutic effect of ASA, being mainly associated with a prothrombotic and/or inflammatory state [65, 94].

3.1.1.2. Genetic causes

Genetic factors also appear to modulate the response to aspirin and, therefore, contribute to AR development [95]. The role of genetic determinants to AR has been proposed by several studies and there is growing evidence reporting that aspirin sensitivity may be affected by genetic polymorphisms [96, 97]. Genetic polymorphisms, one of the most common genetic alterations in the human genome, are defined as DNA sequence variations whose minor allele is present in at least one percent of the general population [98]. About 90% of polymorphisms arise as single-nucleotide polymorphisms (SNPs), the simplest form of these alterations, characterized by the substitution of a single nucleotide in the DNA sequence [98, 99]. SNPs may be spread throughout the entire genome and their biological effects vary depending on their location, ranging from silent to gene expression or protein changes [100]. Such variants are relatively common in the general population, in opposite to rare genetic variants (mutations) implicated in rarer genetic disorders, which induce a detrimental change to protein function and lead to the illness stage [101].

Therefore, the common disease-common variant hypothesis predicts, as its name suggests, that common disorders are likely caused by genetic variants that are also frequent in the general population [102]. As a consequence, common SNPs have a low penetrance and the total genetic risk associated with common genetic variants must be spread throughout several genetic factors [103].

Genetic polymorphisms: Underpinning genetic association studies

In the forefront of genetic association studies are candidate gene analyses, performed to identify and evaluate genetic variants suggested to be associated with a particular trait or disease [104]. Briefly, this approach is based on the selection of a candidate gene with a putative relevant mechanism, followed by the assessment and selection of polymorphisms that may influence a particular trait/disease [105, 106]. Hereinafter, genetic variants are tested for their association with the phenotype of interest, usually through the implementation of a case-control design, in which the allelic frequencies of the selected markers are estimated in a group of test subjects with (cases) and without the disease (controls).

In other perspective, genome-wide association studies (GWAS) have emerged in the last years with the purpose to analyze the association between thousands of genetic variants, namely SNPs, with a specific trait, in a large number of individuals. This approach has radically changed genetic and molecular research, allowing the interrogation of the entire human genome unrestricted by prior hypotheses and with high levels of resolution, which were previously unattainable [107-110]. Regarding their study design, GWAS take advantage of the existence of the principle of linkage disequilibrium (LD), which represents the non-random association between alleles at different loci. It is well established that a large number of SNPs are transmitted across generations in haplotype blocks and the vast majority of the genetic variation present in each block is represented by particular SNPs, referred as tagSNPs [111]. Thereby, LD allows the selection of a lower number of markers to characterize the totality of the allelic diversity presented in each region, being generally reported using a statistical measure of correlation, r^2 (varies from 0 to 1) [112]. High r^2 values reflect a strong linkage between two SNPs, i.e., one allele of the first SNP is commonly inherited together with another allele from the second SNP, being typically only necessary to genotype one of these variants. As consequence, the presence of LD may originate two alternative outcomes: (1) the causal SNP is directly genotyped and associated with the trait of interest, or (2) the identified SNP may not be directly related to phenotype, being in high LD with functional SNP [101]. Thereby, a significant SNP reported by GWAS may not be

considered as the causal variant for the studied trait, rising the need of further evaluations to precisely locate the functional marker [113].

Notwithstanding the audacious study design and the promising rationale underlying this technique, GWAS face some methodological issues. Firstly, due to the analysis of millions of common variants with small effect sizes, these studies require the inclusion of a large number of samples in order to achieve enough statistical power [114]. Moreover, as each tested SNP has an inherent false-positive probability, and considering the number of markers screened in parallel, the cumulative likelihood of a false-positive association is considerable. Thus, a stringent statistical threshold is needed to be established in order to mitigate the issue of multiple comparisons. In accordance, the Bonferroni correction is commonly applied, whereby the generally established significance level of 0.05 is adjusted for a million tested markers. Thereby, a *P*-value of 5.0×10^{-8} has been commonly accepted as the genome-wide significance threshold [115].

Moreover, the high statistical burden associated with this type of studies fosters the need to evaluate GWAS results, in order to discriminate between the false-positive findings and the true associations, through successive replication/validation analyses [116, 117]. Despite being used as synonymous terms, validation and replication are two different concepts, essentially distinguished by the population from which its sample set is stemmed from and/or by the methodology applied: in a replication analysis, both the original and confirmation samples are originated from the same population, whereas to validate GWAS findings, an independent sample set (preferably from a different ethnicity) should be included and/or different methodologies should be used [114, 116].

Candidate gene and GWAS have been driving research in genetic and genomic domains, particularly in the era of personalized medicine, each one associated with advantages and disadvantages (Table 4). Briefly, GWAS allow a holistic discovery of novel genes and pathways related to particular traits or diseases, which, sometimes, might have a direct clinical utility [118]. On the other hand, candidate gene studies are based on the prior knowledge regarding the biological impact of particular genes and pathways, prioritizing and testing those with a putative relevance on the studied phenotype [114]. Thereby, the results reported on both these analyses may complement or confirm each other, making even them complementary approaches in some cases [119].

Table 4 - Comparison between candidate gene and genome-wide approaches.

Candidate gene studies	Genome wide Association Studies (GWAS)
Need a priori hypothesis about the disease mechanism	Hypothesis-free or hypothesis generating approach
Limited number of studied genetic variants (one to hundreds)	High number of studied genetic variants (hundreds of thousands to millions, with imputation)
Limited sample size (usually hundreds)	Large sample size (hundreds to hundreds of thousand)
Low genetic coverage	High genetic coverage (up to 80% of the genome)

Over the last decades, numerous candidate gene and some GWAS have been performed to identify genetic determinants associated with AR (Figure 3) [120]. However, it should be noticed that not all studies referred in this review directly address this phenotype, mainly due to its poorly defined concept. In these cases, either the low or non-response to aspirin or the platelet response/reactivity in patients treated with ASA are often considered in an attempt to address this feature [121]. Among genes reported in the literature as associated with low/non-response to aspirin are included:

PTGS1

Genetic variability in the *PTGS1*, the gene that encodes the COX-1 enzyme**,** is of particular importance due to its preponderant role for aspirin mechanism of action. Therefore, the presence of individual SNPs or specific haplotypes in *PTGS1* might affect enzyme levels or activity and, consequently, its interaction with NSAIDs, namely aspirin [97]. Specific variations in *PTGS1* appear to regulate AA-induced platelet aggregation and thromboxane production, ultimately affecting the efficacy of this antiplatelet drug. Thus, if they confer an increased COX-1 activity, these alterations may contribute to a low or non-response to ASA, leading, eventually, to AR [122].

Specifically, Haluska and colleagues (2003) reported two *PTGS1* SNPs, rs10306114 (A-842G) and rs3842787 (C50T), in complete LD, that have an impact in AA-induced platelet aggregation. They observed that heterozygous carriers for the haplotype of these two markers showed a significantly increased inhibition of PGH2 when compared to homozygous individuals (P=0.010) [123]. This was consistently observed in CVD patients, namely being observed that carriers of the GCGCC haplotype (-842G) and also carriers of the less common 50T allele (P17L) in signal peptide (due to complete LD) showed a significantly decreased sensitivity to aspirin (P=0.009), as determined by AA-induced platelet aggregation [122]. In agreement is also the study developed by Lepntalo *et al*. (2006), in which rs10306114 G allele carriers exhibit a lower response to aspirin

(*P*=0.017) [124]. Despite several studies support the association between rs10306114/rs3842787 with a low response to aspirin, other studies did not confirm their role in as AR genetic predictors [125, 126].

PTGS2

As mentioned, COX-2 plays a major role in inflammation and is seldom expressed under normal conditions in most cells, contrary to COX-1. However, when activated within macrophages, monocytes, and endothelial cells, COX-2 can convert AA to TXA2 which affects platelet aggregation [127]. Therefore, a large number of genetic polymorphisms have been reported within the COX-2 gene (*PTGS2*) although only a few of the variants described have a potential impact on enzyme activity and expression and, consequently, in response to aspirin [128, 129]. For instance, Sharma *et al*. (2013) found a significant association between the G-765C polymorphism (rs20417), located in the gene promoter, and the risk to AR in a population of ischemic stroke patients treated with aspirin. Namely, they showed that the -765C allele may decrease the sensitivity of COX-2 to ASA and contribute to AR development (*P*=0.02, adjusted *Odds Ratio* (aOR), 1.75; 95% CI, 1.06-2.88 and aOR, 3.16; 95% CI, 1.24-8.03 for GC and CC genotype patients, respectively) [129]. A meta-analysis showed significant associations of rs20417 with aspirin insensitivity under both allelic (*P*<0.001; OR, 1.86; 95% CI, 1.44-2.41) and dominant model (*P*<0.001; OR, 1.90; 95% CI, 1.40-2.58). Additionally, in meta-regression analyses, it was observed significant differences in the average platelet number between coronary artery disease (CAD) patients and controls, which might explain the large heterogeneity observed regarding rs20417 analyses (*P*=0.012*)* [130]. Nevertheless, not all studies corroborate these findings [125, 131].

ITGB3

Glycoprotein IIb/IIIa (GP IIb/IIIa), also known as integrin αIIbβ3, is an integrin complex present on the surface of platelets. The GP IIb/IIIa platelet receptors are specific to fibrinogen, a compound involved in platelet aggregation and adhesion [97]. Genetic polymorphisms in *ITGB3*, the gene that encodes GP IIIa, have been associated with differential responses to aspirin therapy, probably leading to an increase in thrombotic events incidence [132]. The rs5918 SNP, leads to the alteration of thymine to cytosine in the exon 2, resulting in the substitution of leucine (P1A1) to a proline (P1A2) at amino acid 33 of the protein [132]. Studies suggest that the presence of rs5918 SNP may be closely related to the aspirin resistance process because the PIA1/A2 alleles may reduce the antiplatelet effects of aspirin and, consequently, elevate the risk to AR [133]. In fact, Scvzeklik *et al*. (2000) noted that carriers of PIA2 allele appear to be more resistant to the

action of aspirin than non-carriers (*P*=0.001) [132]. Additionally, the results of a large systematic review (which include ten studies evaluating rs5918 SNP, namely 4 in healthy individuals and 6 in patients with CVD) showed that the PIA2 allele was significantly related to AR in healthy individuals (*P*=0.009; OR, 2.36; 95% CI, 1.24–4.48). Nevertheless, the effect size of combined analyses between healthy and CVD individuals considerably decrease, which may be explained by the fact that CVD individuals might be taking medication that potentially influences platelet function. In addition, only with LTA assay was possible to observe a correlation between AR and the rs5918 SNP [43]. In accordance is a study performed by Lim *et al*. (2007) in a subset of patients who have been submitted to a cardiovascular bypass and receiving aspirin, which showed that the carriers of PIA2 allele had a consistently more impaired response to ASA after surgery comparing to PIA1 allele homozygous patients. However, the findings do not reach statistical significance (*P*>0.05), which may be justified by the limited sample size [134]. In contrast, some studies state that the reduced response to aspirin might be due to the PIA1 allele or even do not confirm the relation between the rs5918 polymorphism and the insensitivity to ASA [135, 136].

ITGA2 and GP6

Glycoproteins Ia/IIa (GP Ia/IIa or integrin $\alpha_2\beta_1$) and glycoproteins VI (GP VI) are essential collagen receptors. Collagen is involved in the stimulation of platelet activation and aggregation and acts through these receptors [97, 137]. GP Ia/IIa promote the initial interaction between collagen and platelets which are subsequently activated by GP VI [138]. Genetic polymorphisms in *ITGA2* and *GP6* have been proposed to contribute to the reduction of the antiplatelet effect of ASA [139]. When evaluating the role of *ITGA2* rs1126643 (C807T), Su *et al*. (2007) reported that T allele genotype is significantly associated with AR, revealing an almost quadruplicated risk of platelet aggregation during aspirin treatment, in a population of Asian individuals with atherosclerosis (*P*<0.050; OR, 3.76; 95% CI, 2.87–9.58) [140]. This evidence was confirmed on a meta-analysis, in which the estimated risk conferred by rs1126643-T allele reached a value of 2.37 (95% CI: 1.44–3.89; *P*=0.001) for the occurrence of insensitivity to ASA when compared to the alternative allele [130].

The *GP6* rs1613662 (T13254C) leads to a change of serine to proline in the aminoacid sequence and it has been related to coronary thrombus formation [141]. The rs1613662-T allele has also been associated with non-response to aspirin according to PFA-100 laboratory test in patients with CAD (*P*<0.030; OR,5.60; 95% CI, 1.40-22.20) [124]. Notably, the rs1613662 SNP is in complete LD with rs1671152 (which leads to tyrosine to lysine change at amino acid 323) and both have been associated with collagen-induced platelet aggregation, particularly with decreased collagen expression. These conclusions

are based on a genome-wide meta-analysis, in which the rs1671152 was identified. The meta-analysis found a statistically significant association between platelet aggregation and *GP6* rs1671152 in two cohorts of European ancestry individuals (N ≤ 2753 in the Framingham Heart Study, N ≤ 1238 in the Genetic Study of Atherosclerosis Risk) (*P*=4.60 × 10^{-13}; OR, 1.02). Additionally, in an African-American cohort (N ≤ 840 in the Genetic Study of Atherosclerosis Risk) these findings were consistently validated [142].

GP1BA

Von Willebrand factor (vWF) is a large multimeric glycoprotein, expressed in megakaryocytes and the endothelium. This glycoprotein has a key role in the regulation of angiogenesis and in the mediation of platelet aggregation and adhesion, facilitating blood clotting factor VIII [143]. vWF's prothrombotic effects are expressed through a receptor formed by glycoprotein Ib (GP Ib), IX and V. The *GP1BA* encodes the α-subunit of GP Ib, which holds the binding site for vWF and appeared to be highly polymorphic [144]. Namely, the *GP1BA* rs6065 (C1018T) SNP has also been associated with ischemic stroke and with the aspirin response [145]. Fujiwara and colleagues (2007) reported a negative role of the rs6065-C allele in the ASA effectiveness, due to high platelet aggregation (*P*=0.004) [146]. This polymorphism has also been reported in a GWAS meta-analysis performed with the purpose to discover new genetic variants of platelet formation and megakaryopoiesis, in which was analyzed the platelet volume (MPV) and platelet count (PLT) in 66867 European ancestry individuals. The results showed a reliable association of rs6065 SNP with PLT (β=4.191, *P*=2.92 x 10^{-11}) [147]. The same polymorphism had already been highlighted in a previous GWAS which evaluated hematological and biochemical traits in a Japanese population (*P*=2.13 x 10^{-12}; OR,1.13) [148].

TBXA2R

Thromboxane A2 (TXA2) receptor is encoded by the *TBXA2R* gene. TXA2 is a potent vasoconstrictor and platelet activator, whose action is mediated by TBXA2R. The production of TXA2 depends largely on the COX-1 activity, which hence is decreased by ASA [144]. Polymorphisms in *TBXA2R* appear to affect platelet response to TXA2 [149]. Curiously, specific genetic variants also appear to suppress platelet function inhibition caused by the action of ASA. For instance, rs4523-T allele homozygous individuals exhibited reduced aspirin sensitivity (*P*=0.042) [146].

The same association was observed with other *TBXA2R* SNP, rs1131882 (G795A). Specifically, the rs1131882 GG genotype, described more frequently in the aspirin-insensitive group than in the sensitive group (81.8% vs. 62.4%) was considered as a risk factor for AR (*P*= 0.028; OR, 2.71; 95% CI, 1.08-6.81), being related with an elevated level

of AA-induced platelet aggregation [150]. Previous studies in diabetes patients receiving ASA had already shown similar findings (*P*=0.02). Nevertheless, rs4523 and rs1131882 seem to exhibit a significant LD (r^2=0.375) [151].

P2RY1 and P2RY2

ADP is a relevant platelet function mediator whose action is regulated by its binding to the G protein-coupled P2Y receptors, present mainly on the platelet surface. P2Y1 and P2Y12 (encoded by *P2RY1* and *P2RY12* genes, respectively) are the two most well-characterized isoforms (of eight) of the P2Y receptor, able to affect platelet shape and aggregation upon ADP binding [97]. Accordingly, it has been proposed that certain genetic variants in these genes may also influence aspirin efficacy [138]. The rs1065776 SNP, which comprises Thymine for Cytosine base alteration at position 893 of the *P2RY1* gene (C893T), seems to attenuate the effect of ASA in Caucasian patients with a history of myocardial infarction who underwent planned percutaneous coronary intervention (OR, 2.72; 95% CI, 1.12-6.57; *P*=0.03) [152]. The inadequate platelet response to aspirin has also been associated with the *P2RY1* rs701265 SNP (A1622G) in Caucasian patients with stable CAD (OR, 8.53; 95% CI, 1.37 - 53.35; *P*=0.022) [153]. In addition, several SNPs in the *P2RY12* gene have been related to on-ASA residual platelet reactivity (RPR) in patients with CAD, including rs1491974, rs3732765, rs10513398, rs10935841, and rs9859538 polymorphisms. Interestingly, the criteria used in this study to define RPR are in accordance to those elaborated by Gum *et al.* (2012) to diagnose AR [154].

In addition to the aforementioned genetic variants, polymorphisms in other hemostatic proteins have been identified, namely in the platelet endothelial aggregation receptor-1 (*PEAR1*), platelet-activating factor acetylhydrolase (*PLA2G7*) and coagulation factor (F) XIII (*F13A1*) [144, 146, 151, 155, 156]. The *PEAR1* is a gene expressed in megakaryocytes and platelets, whose receptor is involved in megakaryopoiesis and platelet activation [157, 158]. *PEAR1* gene polymorphisms have been strongly associated with higher platelet aggregation even in the presence of aspirin treatment, as the case of rs12041331 (A/G). Carriers of the rs12041331 intronic variant exhibit *PEAR1* overexpression as well as higher platelet PEAR1 protein content, which suggest a functional role for this variant [159]. A GWAS meta-analysis identified several genetic variants associated with platelet response in two European-Ancestry cohorts, including an intronic variant in *PEAR1* gene, rs12566888 (G/T) for which the T allele was associated with a decrease in aggregation response. Curiously, the SNP rs12566888 was in closed LD with rs12041331 (r^2=0.85) and, in an additional validation study, using an African-ancestry cohort, the rs12041331 G allele was significantly related with greater platelet aggregation

independently of ASA therapy [142, 159]. However, not all studies reveal the same findings than the previously described [150].

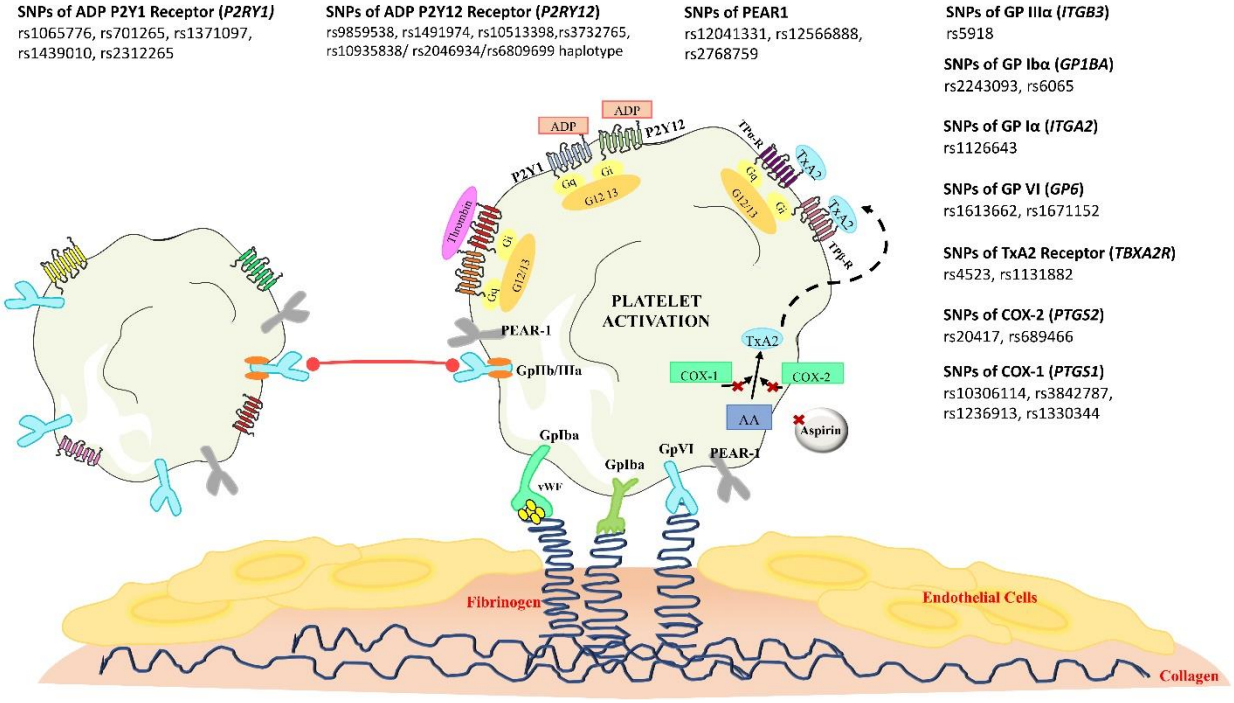

Figure 3 - Overview of mediators and genes (and respective SNPs) reported in the literature as influencing the anti-platelet effect of aspirin. In the figure, the cellular localization of each platelet aggregation-associated mediator is described. Briefly, platelet membrane glycoproteins act as receptors engaged in platelet adhesion. Upon aspirin consumption, COX enzymes are responsible for TXA2 formation on platelets, which is a potent platelet activator. PEAR-1 is a transmembrane receptor whose phosphorylation appears to reinforce the activation of the GP IIb/IIIa fibrinogen receptor and, thereby, the stimulation of platelet aggregation. Nonetheless, the exact function of this receptor is still largely unknown, and its surface ligands are not yet identified. SNPs may affect the structure of glycoproteins or gene/protein content expression and induce an abnormal platelet activity in response to aspirin. In the figure are exposed the main genetic variants which potentially affect the antiplatelet action of aspirin (Adapted from [138]).

Notwithstanding, it is essential to emphasize that additional genes, and related genetic variants might be associated with an inadequate response to ASA. However, due to inconsistent AR definition and AR-associated results, this chapter's aim was to focus on genetic variants that evidence more relevance in the literature. Nevertheless, to the best of our knowledge, in Supplementary Table 1, all the polymorphisms found to be studied in the AR context are exhibited.

The reasons behind the disagreement in study findings are not entirely clear, but likely reflect the lack of precise and standardized criteria to define AR, the AR inadequate assessment due to the different methodologies currently used to evaluate platelet function,

usually with poor correlation between them and not specific for AR. Thus, it is crucial to ascertain which of the reported assays best represents the ASA effect or even to developed new assays able to fill the limitations of previous ones, i.e., AR-specific, cost-effective, reproducible and correlated with future cardiovascular events [90]. In addition, patient's heterogeneity observed between studies, namely according to gender (some studies performed only with male patients), ethnicity (strong associations were more commonly found in Asian than European population), age (as some studies used a sample with a restricted range of ages, focusing only on the young (20-30 years) or older classes (>60 years) and sometimes the limited sample size may also contribute to the unreliable and conflicting results reported [43].

3.1.2. AR clinical management

Being the AR a multifactorial pathological condition, it is important to take into account which potential factors predispose specific individuals to the development of a low or non-sensitivity to aspirin therapy [127]. In some patients, the discontinuation of concomitant medication that interacts with ASA or the emphasis on the need to aspirin treatment adherence might be enough to overcome this issue. However, in patients with good treatment adhesion and that do not take interacting medication, the aspirin non-response management is less clear. Dose and frequency adjustments might be considered, although there is no evidence that higher doses of aspirin are more effective than lower doses [90, 160]. Additionally, in the attempt to overcome AR manifestation, it may be helpful the concomitant use of aspirin (or even its replacement) with, for instance, clopidogrel (a P2Y12 receptor antagonist), ticlopidine, GPIIb/IIIa receptors inhibitors or other antiplatelet drugs to ensure an adequate inhibition of platelet function [127].

Nevertheless, there are still no well-defined and specific strategies and guidelines to treat these patients (Figure 4) [68].

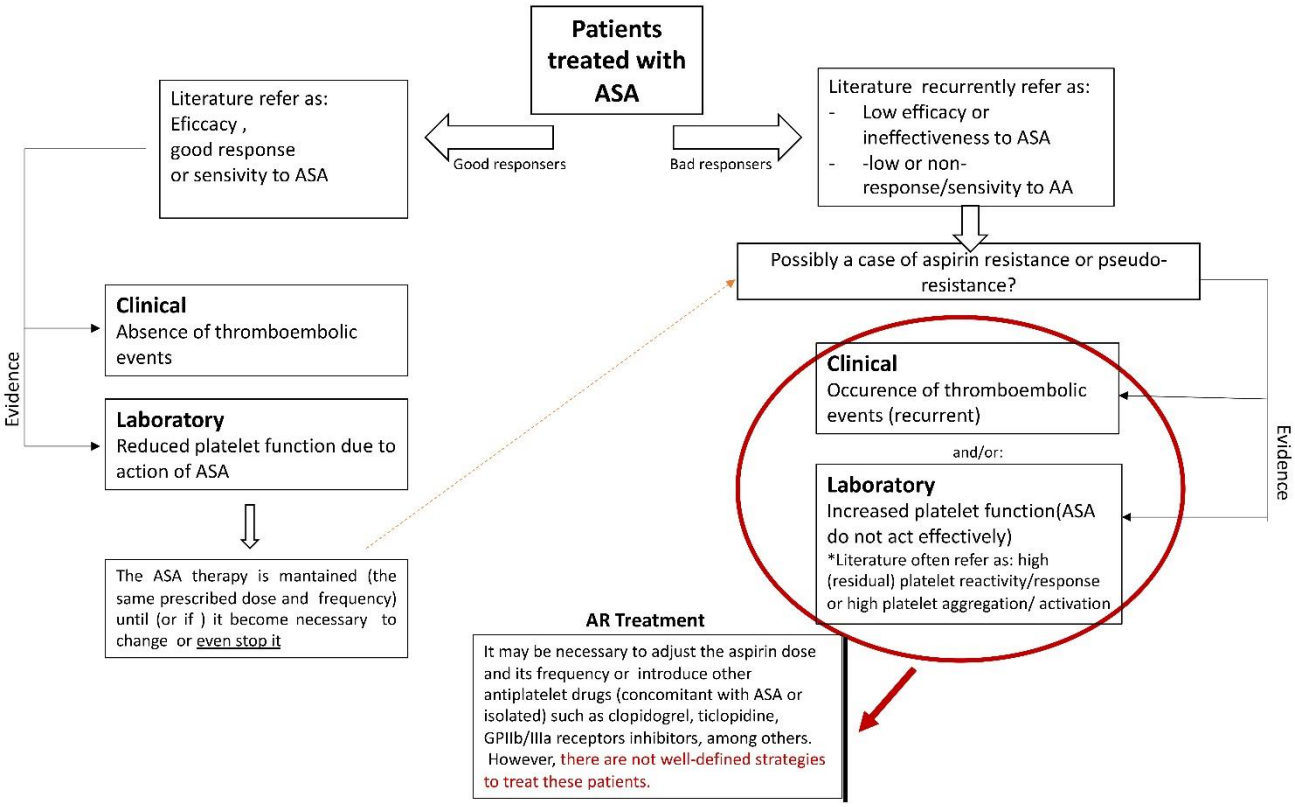

Figure 4 - Overall clinical management of patients treated with aspirin, according to their response to this antiplatelet drug. The insufficient response to ASA may result in aspirin resistance (AR) or pseudo-resistance. The AR-clinical and laboratory evidence are the occurrence of thromboembolic events and increased platelet function, respectively. In this context, it may be helpful the adjustment of ASA dose or its frequency, or its concomitant use (or even its replacement) by other antiplatelet drugs, such as clopidogrel, ticlopidine, GPIIb/IIIa receptors inhibitors, among others. The therapy with ASA is maintained for good-responders until or if it becomes necessary to change or withdraw it. If the reason behind the medication adjustment is the development of resistance, the clinical management for these patients is the same that previously described.

Due to the essential role of ASA as inflammatory inhibitor, the AR manifestation might potentiate the inflammatory process and contribute to the development, exacerbation, and prognosis of several pathologies, as previously described. Following the extensive evaluation within CVDs, the role of aspirin and AR should also be further exploited in oncology, namely in OC patients due to the preponderant role proposed for inflammation regarding this gynecological malignancy [161-163].

Chapter 4: Ovarian cancer overview

OC is the eighth most common cancer among women worldwide, being estimated 296 000 newly diagnosed cases in 2018 (3.4 % of cases by cancer in women), with more

than 90% having epithelial origin. Regarding mortality rates, OC also represents the eighth most frequent cause of death by cancer, with approximately 185 000 associated deaths registered in the same year (4.4% of deaths by cancer in women) [4]. In Portugal, in the same year, it was estimated that about 570 new OC cases occurred with approximately 400 deaths, showing an age-standardized incidence and mortality rates of 7.0 cases and 4.3 deaths/100 000 women, respectively [164].

Based on the World Health Organization (WHO) criteria for gynecological tumors, epithelial ovarian cancer (EOC) can be subdivided into seven histological subcategories, which is illustrative of the heterogeneity associated with this disease. Among them are included: serous, mucinous, endometrioid, clear cell, Brenner, seromucinous and undifferentiated histologic subtypes [165]. Additionally, all mentioned histologic subtypes, with exception of undifferentiated type, may also be characterized regarding their behavior and, thus, be considered as benign, borderline or malignant [166, 167]

Due to the different cellular, molecular and clinical features but also the distinct treatment patterns of OC histological subtypes, the accurate staging of this disease becomes essential for successful disease management. Thus, based on the International Federation of Gynecologists and Obstetricians (FIGO) guidelines, OC may be classified according to its dissemination pattern at diagnosis: stage I tumors are limited to ovaries or fallopian tube(s); stage II tumors include one or both ovaries or fallopian tubes with pelvic extension or primary peritoneal tumors; stage III tumors involve one or both ovaries or fallopian tubes, with cytologically or histologically confirmed dissemination to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes; stage IV include those with distant metastasis, excluding peritoneal metastases [167, 168]. These criteria provide not only essential information about the histopathology of the disease but also reflect OC dissemination patterns. In turn, this facilitates the assessment of patients' prognosis, while also serving as an auxiliary tool to the planning, implementing and evaluation of the most suitable treatment option, prompting a better management of the disease [169].

OC dissemination may occur through several propagation pathways, namely, lymphatic (through lymph nodes), hematogenic (up to parenchyma of distant organs, mostly to liver and lung), transcavitary and contiguous. Preferentially, OC dissemination occurs through transcavitary route, i.e., from primary organ up to peritoneal cavity by transperitoneal migration, exfoliation, and malignant cell deployment [170, 171]. This is the most clinically relevant propagation route, being an early phenomenon in the natural history of the disease which, in the large majority of cases, has a significant impact on prognosis [172]. Due to the predilection for the transcavitary route and its early stage indolent nature, numerous organ systems are already compromised at OC diagnosis [173]

Even though the several hypotheses proposed to characterize the ovarian carcinogenesis and etiology, age is considered as the major determinant for OC development as nearly 80% of OC cases are diagnosed after the age of 45 years, affecting mostly menopausal women [174, 175].

Individual genetic background also reveals one of the most significant and consistent risk factors for OC, being responsible for around 10-20% of all cases [176]. Most commonly associated with hereditary OC are germline mutations in *BRCA1* (3-6%) and *BRCA2* (1-3%), associated with hereditary breast/ovarian cancer syndrome, which account for a lifetime risk of OC development of 40% and 20%, respectively, when compared with the risk of 1.4% for general population [177, 178]. Less common causes of inherited OC comprise mutations in the mismatch-repair (MMR) *MSH2*, *MLH1*, *MSH6,* and *PMS2* genes, associated with the Lynch Syndrome, which contribute to 2-4% of OC cases [177, 179, 180]. More recently, new susceptibility OC genes like *BRIP1*, *RAD51C*, *RAD51D*, or genes related to the Fanconi anemia pathway have been identified. Apart from mutations in *BRCA1* and *BRCA2*, the remaining mentioned mutations are individually rare, however once combined might be responsible for a substantial fraction of cases [181]. Furthermore, nulliparity, late menopause (>52 years), early menarche (<12 years), and hormone replacement therapy are some of the reproductive and hormonal factors which might also potentiate the risk for OC. Still, geographic and lifestyle factors are also considered to have an impact on the increased risk to OC [182].

Concerning OC treatment, substantial advances have been reached over the last decades, namely with the adoption of molecular therapies targeting the inhibition of angiogenesis and DNA repair (bevacizumab and poly ADP ribose polymerase (PARP) inhibitors, respectively). Nevertheless, as standard first-line treatment for EOC is established the combination of cytoreductive surgery, followed by adjuvant chemotherapy with the platinum (carboplatin or cisplatin) and taxane (paclitaxel or docetaxel) duplet, in each 21/21 day for 6 cycles [183, 184]. Despite EOC be considered a chemosensitive neoplasia, with about 40-60% of complete response rates in advanced disease stages, up to 75% of patients will relapse becoming potential candidates to second-line chemotherapy.

Due to the lack of effective screening strategies and to an indolent biological behavior, most of EOC cases are frequently diagnosed in advanced stages of the disease [185]. Concomitantly with the manifestation of a resistant treatment phenotype, EOC represents the most lethal gynecological malignancy, with 5-year overall survival (OS) rate around 45% [186-189].

A substantial number of clinicopathological factors have already been implicated in EOC prognosis. Tumor size, disease stage, histological subtype, differentiation degree and the extent of residual disease after surgery are considered as key prognostic factors for

EOC. In contrast, the identification and characterization of predictive biomarkers for EOC first-line treatment have proven to be a challenge, which demonstrate the need of more research in this field [190]. The minimal improvement in mortality rate over the years, the indolent and inflammatory nature, the growing evidence showing the protective role of aspirin in this field and the lack of predictive and prognostic biomarkers described, makes of OC an attractive tumor model to implement in this study.

2. Aims

2.1. Main Aim

The main objective of this work was to evaluate the impact of aspirin resistance (AR)-associated genetic variants in the clinical outcome of epithelial ovarian cancer (EOC) patients.

2.2. Specific Aims

- Perform a literature review regarding aspirin and associated resistance mechanisms, with focus on genetic association studies.
- Selection of genetic variants reported to be associated with AR.
- Assess the influence of selected AR-associated genetic variants in the clinical outcome of a cohort of EOC patients.

3. Materials and Methods

3.1. Study population description

A retrospective hospital-based cohort study was performed on histologically confirmed EOC patients admitted, between January of 1996 and December of 2017, in the departments of gynecology and oncology of the Portuguese Institute of Oncology, Porto, Portugal (IPO-Porto). Within this group of patients were excluded those who had less than 18 years old, who were only admitted for a second opinion or to be subject of specific treatment techniques, such as hyperthermic intraperitoneal chemotherapy or even with follow-up in other institutions. Consequently, a cohort of 336 consecutive conveniently sampled patients from the North region of Portugal and for whom biological material was available was enrolled.

Tumor staging was reported according to the FIGO guidelines and the tumor response to chemotherapy was assessed based on RECIST criteria [191, 192]. Patient's clinicopathological data and follow-up were acquired from their medical records. The mean age of selected EOC patients was 55 years (median = 55 years; minimum = 21 years; maximum = 80 years), from which 51.8% were post-menopausal women and mostly diagnosed with advanced stage disease (FIGO III/IV; 60.7%). Concerning the extent of residual disease, the distribution was as follows: optimal surgery resection was observed in 51.3 % of the cases whereas 2.9% and 42.5% presented residual disease <1 cm and ≥1 cm, respectively (no available information for 3.3% of patients). Concerning the histological subtype, 56.8% of the patients presented tumors with serous differentiation, 12.5% clear cell, 9.8% mucinous, 10.1% endometrioid, and the remaining 10.8% less common histological subtypes. Considering the adopted therapeutic strategy, 88.9% of patients were submitted to the standard regimen, i.e., to the cytoreductive surgery followed by a combination of Paclitaxel (175mg/m^2) and Cisplatin (75 mg/m^2) or Carboplatin (Area under the curve 5-7.5), although dose adjustments were required whenever severe toxicity was reported. Chemotherapy alone (2.7%), neoadjuvant chemotherapy (5.7%) or only surgery (1.2%) were also taken into account as first-line treatment options.

Follow-up data were reviewed from the initial diagnosis through July 2018 in 322 patients (96% of all patients). The median follow-up was 144 months (CI 95%, 132.00-163.00 months).

Prior to the inclusion in the study, written informed consent was obtained from each participant, based on Helsinki Declaration principles. Furthermore, this study was approved by the ethics committee at IPO-Porto (CES-IPO: 286/2014).

3.2. Laboratory procedures

3.2.1. Sample collection and genomic DNA extraction

Peripheral venous blood samples were obtained with a standard technique and collected in ethylenediamine-tetraacetic acid (EDTA)-containing tubes.

From blood samples was extracted genomic DNA using the extraction kit Qiagen®, QIAmp DNA Blood Mini Kit (Qiagen® 51106), as indicated by the manufacturer's procedure.

3.2.2. SNP selection

To select the variants to be evaluated in this study, all polymorphisms associated with AR phenotype were retrieved, both reported by candidate-gene and GWAS. The GWAS reported variants were further submitted to the GWAS4D software, which considers the *P*-value reported by the original study and the possible functional impact of each variant according to its genomic location, in order to prioritize AR-related variants. Thus, based on the 1) priority ranking generate by GWAS4D software; 2) minor allele frequency (MAF) in the Iberian population (>10%); 3) the availability of the respective validated genotyping assay and 4) the putative relevance in AR and cancer biological pathways, SNP selection was performed. Thus, based on indicated criteria, the *PTGS2* rs20417, *TBXA2R* rs1331882, *ITGB3* rs5918, *PEAR1 rs*12041331*, RGS7* rs2502448 and *GSR* rs3779647 SNPs were selected (Table 5).

Table 5 - Genetic polymorphisms reported to affect the response to aspirin.

Gene	Gene product	Biological Function	refSNP (rs number)	Variation/ Aliases	MAF[1]		SNP location	Putative functional effect	Genetic association study [REF]
					EUR	IBS			
PTGS2	COX-2	COX-2 is involved in the conversion of AA into TXA2, which affect platelet aggregation. This enzyme is only activated under inflammatory states	rs20417	G-765C	0.151	0.150	NC transcript exon	C allele is associated with a lower transcriptional activity of COX-2	Candidate gene [129, 130]
ITGB3	GPIIIa	GP IIb/IIIa platelet receptors are specific to fibrinogen, having a role in platelet aggregation and adhesion	rs5918	T1565C (PlA1/A2)	0.132	0.136	Missense	It leads to de alteration from leucine to proline in aa sequence. Considered to affect splicing regulation by decreasing the exon splicing enhancer binding motif in the coding sequence containing the same protein domain	Candidate gene [43]
TBXA2R	TXA_2 receptor	TXA_2 receptor interacts with thromboxane A2, being involved with platelet aggregation and with the regulation of hemostasis	rs1131882	G795A	0.147	0.173	Missense	A allele may affect the transcription and/or translation efficiency of both isoforms of the TBXA2R gene. This SNP might be in LD with one or several SNPs in the gene promoter region, intronic silencer or enhancer region.	Candidate gene [150]
PEAR1	Platelet endothelial aggregation receptor-1	The receptor encoded are involved in megakaryopoiesis and platelet activation	rs12041331	A>G	0.092	0.145	Intronic	G allele is associated with an increased PEAR1 expression as well as platelet PEAR1 protein content	Candidate gene/GWAS [142, 159]
RGS7	Regulator of G-protein signaling 7	Regulates G protein-coupled receptor signaling cascades	rs2502448	T18097C	0.401	0.379	Intronic	The SNP may potentially affect receptor function through alternative splicing mechanisms. Also, it may be in LD with promoter SNPs which have not yet been identified or the SNP.	Candidate gene/GWAS [193, 194]
GSR	Glutathione reductase	Reduces oxidized glutathione in the cytosol, being also involved in preventing the accumulation of hydroperoxides and plays a role in the AA metabolites formation	rs3779647	C-130T	0.428	0.467	Intronic	C allele may be associated with increased glutathione circulation levels	Candidate gene/GWAS [193, 194]

Abbreviations: aa, amino acid; COX, cyclooxygenase; EUR, European; GP, glycoprotein; IBS, Iberian; LD, Linkage Disequilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism, TX, thromboxane, TXA2, thromboxane A2, NC, non-coding; [1]Data obtained from Ensemble Database.

3.2.3. Polymorphisms genotyping

Polymorphism genotyping for all selected SNPs was performed using TaqMan® Allelic Discrimination methodology (Figure 5) through the Real-Time Polymerase Chain Reaction (PCR) technique. The features related to the six tested assays are available in Table 6.

Table 6 - Characterization of the six tested assays used in this study.

SNP ID	Gene	Assay ID	VIC	FAM	DNA flanking sequence
rs20417	*PTGS2*	C__11997909_40	C	G	GAGG**[C/G]**GGGAAAGGTAAATTCTCCTCATAAT
rs1131882	*TBX2AR*	C__2576300_10	A	G	GCGGGTTTCGCAGCACTGTCTGGGC**[A/G]**ATGAAGACCTGCAAAGGGGAGAGCT
rs5918	*ITGB3*	C__818008_30	C	T	GCTCCTGTCTTACAGGCCCTGCCTC**[C/T]**GGGCTCACCTCGCTGTGACCTGAAG
rs12041331	*PEAR1*	C__31432615_10	A	G	AAGTCCCTTCTGCTGTCTCACTTCC**[A/G]**TCACCCTTACTCTCTGCTTTCTATA
rs2502448	*RGS7*	C__26887460_10	C	T	AGTACAGGACCTAACACAATATAGC**[C/T]**ATACAACAACAATAAAAATGTTAGC
rs3779647	*GSR*	C__25472374_10	C	T	GTTTGCTGATGCCAACACAATTCTC**[C/T]**GTTTTTCAAGTTTCTGTAGAACTTC

Real-time PCR reactions were performed using 6 µL reaction mixture, encompassing the following components: 2.5 µL of TaqPathTM ProAmpTM Master Mix (1x), 0.125 µL of TaqMan® SNP Genotyping Assay mix, 2.375 µL of sterile water and 1 µL of genomic DNA. Thermal conditions were based on the activation of Taq DNA Polimerase at 95°C for 10 minutes, followed by 45 cycles at 92°C for 15 seconds with the purpose to denature DNA chain and 60°C for 1 minute to primers pairing and extension.

The StepOne Plus Real-Time PCR system and StepOne Software (version 2.3 Applied Biosystems) were used to detect amplification and analyze the data. To certify the quality of genotyping, two negative controls were considered in each amplification reaction (to prevent false positives) and double sampling was performed in, leastwise, 10% of the samples, with an accuracy over 99%. The evaluation of genotype results was independently performed by two researchers, without any knowledge about the patient clinical status.

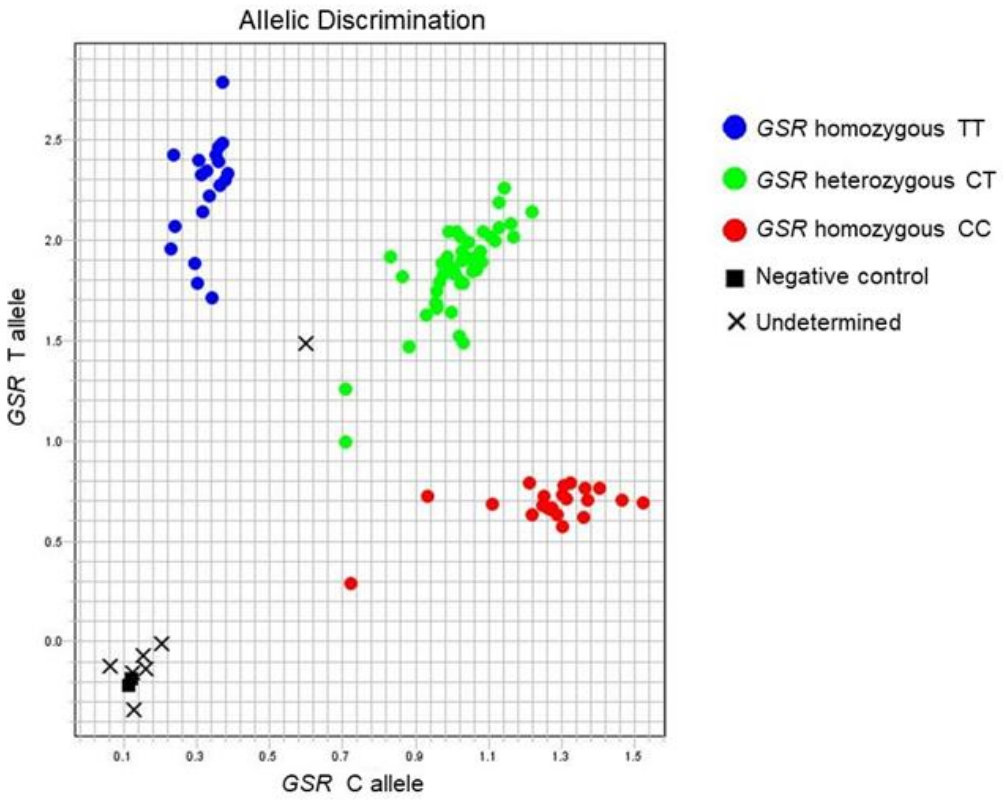

Figure 5 - Exemplification of an allelic discrimination plot for *GSR* rs3779647 polymorphism.

3.3. Statistical analysis

Statistical analysis was performed using the computer software IBM® SPSS® Statistics for WindowsTM (version 24.0, SPSS Inc, 2016).

Chi-square test ($\chi 2$) and Student's *t*-test were used to evaluate associations between polymorphisms and patient's clinicopathologic features for categorical or continuous (age) variables, respectively.

Kaplan-Meier method was used to obtain survival curves. The curves were examined through the log-rank test, a statistical test for equality of survival distributions. For each variant was established the adequate genetic model after an initial comparison between Kaplan-Meier curves according to the log-additive genetic model. The stratification of subgroup was also performed concerning the FIGO stage (FIGO I/II vs. III/IV) and the extension of surgical resection (complete or <1cm vs. others).

Overall survival (OS) at 5 years was described as the percentage of patients alive after 5 years of diagnosis and represented the primary outcome. Disease-free survival (DFS) at 5-years was established as the secondary outcome and was defined as the 5-year period from the date of diagnosis until the date of first relapse or last clinical assessment in

patients that show complete response to the first-line treatment. Endpoint definition was based on RECIST criteria [195].

The death and recurrence risks at 5-years were estimated by Cox proportional hazard ratio (HR), along with 95% confidence interval (CI), adjusted for hormonal status (pre- vs. post-menopause), histologic subtype (serous vs. others), age (< 60 years vs. ≥ 60), tumor stage (stage I/II vs. III/IV) and tumor grade (well-differentiated vs. others). The cause of death was obtained from the patient's medical records.

The concordance (*c*)-index was applied to compare the predictive ability of proposed models. The Harrell's concordance indexes were used to determine the predictive value, where c>0.05 was considered a good prediction ability [196]. The Cox regression proportional hazard model for selected polymorphisms was validated recurring to bootstrap resampling to accurate the reliability of estimated risks (1000 replications).

All tests were two-sided, and a level of $P<0.05$ was established as statistically significant.

4. Results

4.1. Descriptive statistics of the selected polymorphisms and the association with clinicopathologic features

The genotype distribution for each genetic variant studied is described in Table 7, along with the respective MAF and genotype failure rate.

Table 7 - Genotype distribution and respective MAF and genotype failure observed for each genetic variant evaluated in this study.

Genetic polymorphism	Genotype Frequency	MAF[1] (allele)	Genotype Failure
PTGS2 rs20417		23% (C)	5.7%
GG homozygous	58.7% (n=186)		
CG heterozygous	36.9% (n=117)		
CC homozygous	4.4% (n=14)		
TBXA2R rs1131882		14% (A)	2.4%
GG homozygous	71.6% (n=235)		
AG heterozygous	28.4% (n=93)		
AA homozygous	0 % (n=0)		
ITGB3 rs5918		15% (C)	3.6%
TT homozygous	71.9% (n=233)		
CT heterozygous	25.3% (n=82)		
CC homozygous	2.8% (n=9)		
PEAR1 rs12041331		12% (A)	3.3%
GG homozygous	78.5% (n=264)		
AG heterozygous	19.7% (n=64)		
AA homozygous	1.8% (n=6)		
RGS7 rs2502448		39% (C)	4.8%
TT homozygous	35.6% (n=114)		
CT heterozygous	50.6% (n=162)		
CC homozygous	13.8% (n=44)		
GSR rs3779647		47% (C)	5.1%
TT homozygous	26.7% (n=85)		
CT heterozygous	51.7 % (n=165)		
CC homozygous	21.6% (n=69)		

Abreviations: MAF, Minor Allele Frequency, [1] MAF observed in the study cohort

In the present study, no significant statistical differences were observed between the genotypes of each selected polymorphism, under the log-additive or dominant genetic model, and the patients' clinicopathological parameters, namely regarding age, hormonal status (pre vs post-menopause), FIGO stage (I/II/ vs III/IV), histological subtype (serous vs others), tumor differentiation grade (well differentiated vs others) and the extent of residual disease (complete or optimal (<1cm) vs others), as described in Table 8.

Table 8 - Association between *PTGS2*, *TBXA2R*, *ITGB3*, *PEAR1*, *RGS7* and *GSR* genotype (minor allele vs reference genotype) and clinicopathological parameters in a cohort of EOC patients.

	Age (years)	**Hormonal status**		**FIGO stage**		**Histological subtype**		**Differentiation grade**		**Extent of surgical resection**	
	mean±SD	**Pre-menopause**	**Post-menopause**	**I/II**	**III/IV**	**Serous**	**Others**	**Well differentiated**	**Non- well differentiated**	**Complete/ Optimal cytoreduction**	**Others**
***PTGS2* rs20417**											
GG genotype	55.8±12.3	58(32.8%)	119(67.2%)	66(36.3%)	116(63.7%)	109(58.6%)	77 (41.4%)	30(20.4%)	117(79.6%)	86(46.9%)	76(46.9%)
CG/CC genotypes	55.3±12.3	51(41.1%)	73(58.9%)	58(44.6%)	72(55.4%)	71(54.2%)	60(45.8%)	21(18.8%)	91(81.2%)	73(61.3%)	46(38.7%)
P-value	0.285	0.137		0.137		0.436		0.740		0.168	
***TBXA2R* rs1131882**											
GG genotype	55.4±11.9	80(35.6%)	145(64.4%)	97(42.0%)	134(58.0%)	136(57.9%)	99(42.1%)	41(21.4%)	151(78.6%)	123(58.9%)	86(41.1%)
AG genotype	55.7±13.4	30(34.5%)	57(65.5%)	29(31.5%)	63(68.5%)	52(55.9%)	41(44.1%)	11(14.3%)	66(85.7%)	40(48.8%)	42(51.2%)
P-value	0.834	0.859		0.082		0.747		0.185		0.119	
***ITGB3* rs5918**											
TT genotype	55.4±12.3	84(37.7%)	139(62.3%)	91(39.6%)	139(60.4%)	139(59.7%)	94(40.3%)	36(18.9%)	154(81.1%)	113(54.3%)	95(45.7%)
CT/CC genotypes	55.5±12.6	24(28.2%)	61(71.8%)	33(37.1%)	56(62.9%)	47(51.6%)	44(48.4%)	15(19,7%)	61(80.3%)	47(59.5%)	32(40.5%)
P-value	0.940	0.121		0.683		0.190		0.883		0.431	
***PEAR1* rs12041331**											
GG genotype	55.6±12.6	81(33.3%)	162(66.7%)	97(38.6%)	154(61.4%)	148(58.0%)	107(42.0%)	39(18.8%)	168(81.2%)	124(54.9%)	102(45.1%
AG/AA genotypes	54.7±11.5	28(42.4%)	38(57.6%)	28(40.6%)	41(50.4%)	39(55.7%)	31(44.3%)	12(20.3%)	47(79.7%)	38(61.3%)	24(38.7%)
P-value	0.585	0.170		0.771		0.727		0.796		0.366	
***RGS7* rs2502448**											
TT genotype	55.3±12.1	42(38.2%)	68(61.8%)	43(38.1%)	70(61.9%)	57(50.0%)	57(50.0%)	21(23.1%)	70(76.9%)	59(60.2%)	39(39.8%)
CT/CC genotypes	55.3±12.5	67(34.5%)	127(65.5%)	81(40.1%)	121(59.9%)	124(60.2%)	82(39.8%)	31(18.1%)	140(81.9%)	101(54.6%)	84(45.4%)
P-value	0.991	0.524		0.721		0.078		0.339		0.365	
***GSR* rs3779647**											
TT genotype	55.5±11.7	27(32.9%)	55(67.1%)	28(34.1%)	54(65.9%)	54(63.5%)	31(36.5%)	17(25.0%)	51(75.0%)	44(57.1%)	33(42.9%)
CT/CC genotypes	55.4±12.6	80(36.2%)	141(63.8%)	95(40.9%)	137(59.1%)	128(54.7%)	106(45.3%	33(17.1%)	160(82.9%)	114(55.3%)	92(44.7%)
P-value	0.901	0.596		0.278		0.159		0.155		0.786	

$\chi 2$ test with the exception of t-student analysis for the age comparison; Abreviations: SD,standard deviation

4.2. Association of selected polymorphisms with the clinical outcome of EOC patients

Concerning survival outcomes for all the patients included in this study, 5-year OS rate was 64.9% (mean 49.35 months; 95% CI, 47.55-51.16 months), holding on 44.5% for the entire follow-up period. Considering time to disease recurrence as endpoint, 5-year DFS rate was 77.0% (mean 54.83 months; 95% CI, 53.53-56.14 months).

4.2.1. SNPs selected from candidate gene studies

As presented in Table 5, the rs20417, rs1131882 and rs5918 genetic variants located in *PTGS2*, *TBXA2R,* and *ITGB3*, respectively, were selected from candidate gene studies.

- **Primary outcome: 5-year OS**

Considering the 5-year survival curves obtained through Kaplan-Meier method and log-rank test, no statistically significant differences were observed on survival according to the log-additive model for *PTGS2* rs20417 and *ITGB3* rs5918 (P=0.653 and P=0.387, respectively), inclusively after stratification according to FIGO stage (P>0.05). In opposite, when applying the dominant genetic model (TT vs CT/CC), *ITGB3* rs5918 SNP was significantly associated with 5-year OS for the subgroup with early disease stage patients (FIGO I/II, P=0.036), as presented in Figure 6. Within this subgroup, TT genotype patients had a reduced survival time when compared with C-allele carrier patients. However, it was not possible to estimate the 5-year risk of death due to the reduced number of cases, and the consequent number of registered events, accounting in this analysis.

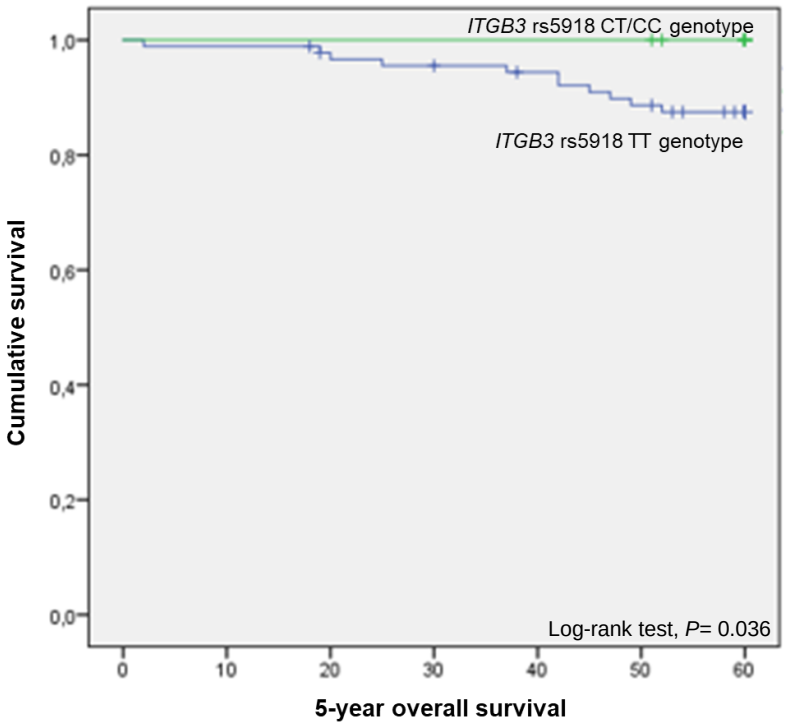

Figure 6 - Overall survival at 5-years by Kaplan-Meier and log-rank test for the subgroup of EOC patients with FIGO I/II disease stages at diagnosis, according to *ITGB3* rs5918 polymorphism genotypes (dominant genetic model). The group of patients carrying the TT genotype had a significantly lower survival when compared to patients with C allele genotypes (CT/CC genotypes) (*P*=0.036).

Regarding *TBXA2R* rs1131882 polymorphism, it is important to highlight that AA genotype patients were not observed on the entire cohort and, for this reason, only AG and GG genotype patients were included for analysis. Nevertheless, no significant differences in 5-year OS were observed for the overall cohort (*P*=0.316) according to *TBXA2R* rs1131882 genotypes. However, when considering a subgroup analysis restricted to early disease stage patients (FIGO I/II) who were submitted to incomplete/sub-optimal cytoreduction, a significantly improved survival was observed for *TBX2AR* rs1131882 GG genotype patients when compared to heterozygous patients (*P*=0.029; mean of survival time, 55.6 and 45.50 months, respectively). Regarding the subgroup of patients with advanced disease stages (FIGO III/IV), even when stratifying by the extension of surgical resection, no statistically significant associations were verified on 5-year OS (*P*=0.838 and *P*=0.779 for complete and incomplete surgical resection, respectively). Nevertheless, it should be highlighting the limited sample size observed in the subgroup analyses, which inclusively impairs a proper risk of death estimation (Table 10).

- **Secondary outcome: 5-year DFS**

Concerning the influence of *PTGS2* rs20417 and *ITGB3* rs5918 in 5-year DFS for the whole cohort, no statistically significant associations were found, even considering both the log-additive and the dominant genetic models *(P*>0.05). In addition, even stratifying the analysis according to the FIGO stage/extension of surgical resection, and independently of the genetic model adopted, no statistical association reached a significant value.

The lack of statistically significant associations was also observed regarding the *TBXA2R* rs1131882 variant for the overall cohort (*P*=0.304). However, for early stage patients with residual disease above 1 cm, we observed that GG genotype patients had a prolonged time until disease recurrence when compared to AG genotype patients (*P*=0.002). Nevertheless, this result is limited by the low number of patients included in each subgroup [197].

4.2.2. SNPs selected from GWAS

The genetic variants rs12041331, rs2502448, and rs3779647 located in *PEAR1*, *RGS7,* and *GSR*, respectively, were selected from GWAS. Nevertheless, some of these variants were further evaluated in candidate gene studies.

- **Primary outcome: 5-year OS**

Regarding the impact of selected *PEAR1, RGS7 and GSR* polymorphisms on 5-year OS, no statistically significant associations were observed, under the log-additive model (*P*=0.705, *P*=0.727*, P*=0.893, respectively*)*. However, upon stratified analysis, significant differences in survival at 5-years in the subgroup of early disease stage patients who were submitted to incomplete/suboptimal surgical resection were observed according to the *RGS7* rs2502448 genotypes (*P*=0.035). Namely, TT genotype patients presented a significantly decreased survival time when compared to the C allele carriers (*P*=0.010, mean of survival 37.00 and 57.75 months, respectively).

A significant association for the *GSR* rs3779647 polymorphism was also observed for advanced disease stage patients (FIGO III/IV) submitted to incomplete/suboptimal surgery (*P*=0.024). As reported in Figure 7, C allele carriers showed a significantly diminished survival time when compared to TT genotype patients (*P*=0.010; mean survival 40.21 and 49.23 months, respectively). Furthermore, among patients considered as platinum-sensitive, C allele carrier patients had a significantly reduced of survival time than

TT genotype patients (*P*=0.036, mean of survival 52.89 and 58.71 months, respectively), as showed in Figure 8.

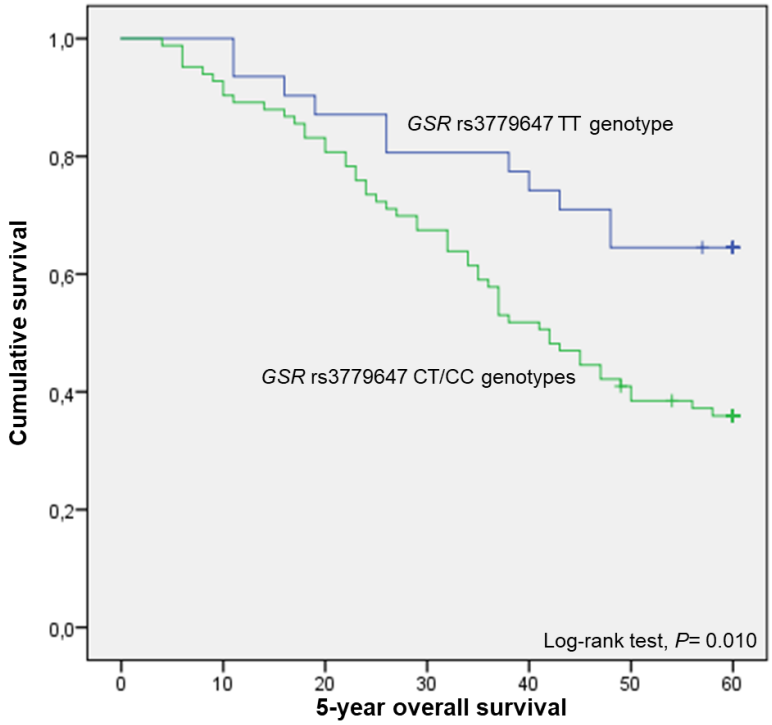

Figure 7 - Overall survival at 5-years by Kaplan-Meier and log-rank test for the subgroup of EOC patients with FIGO III/IV stages disease at diagnosis and submitted to incomplete/suboptimal surgical resection, according to GSR rs3779647 polymorphism genotypes (dominant genetic model). The group of patients with CT/CC genotype had significantly lower survival when compared with patients TT genotype, (*P*=0.010).

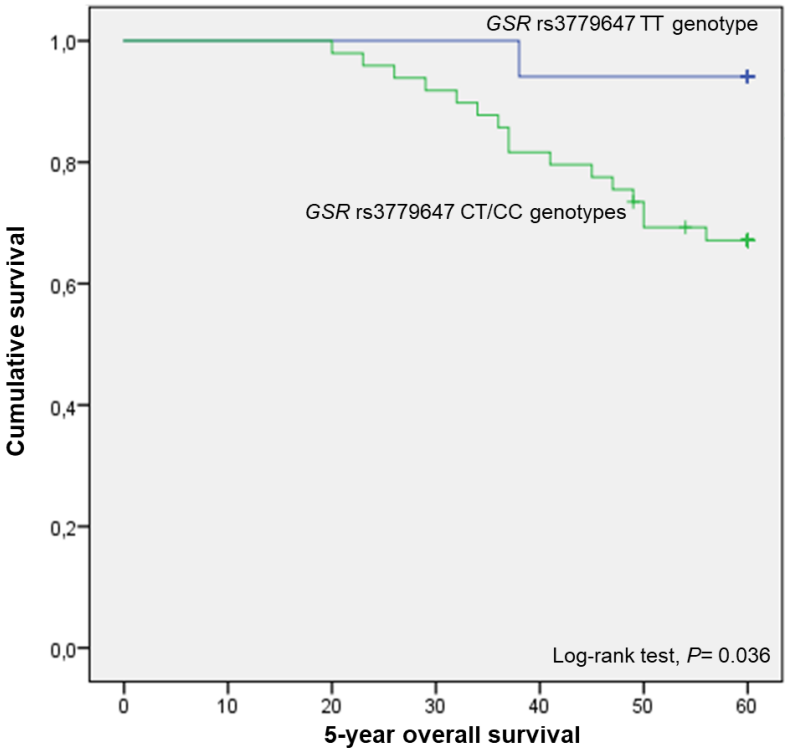

Figure 8 - Overall survival at 5-years by Kaplan-Meier and log-rank test for the subgroup of platinum-sensitive EOC and submitted to incomplete/suboptimal surgical resection, according to *GSR* rs3779647 polymorphism genotypes (dominant genetic model). The group of patients with CT/CC genotype had significantly lower survival when compared with patients TT genotype (*P*=0.036).

In a further step, applying a multivariate Cox-regression model, we evaluate the influence of several EOC prognostic determinants and *GSR* polymorphism in the 5-year risk of death estimation among the subgroup of patients submitted to incomplete/ suboptimal surgical resection (Table 9). These results were complemented with the inclusion of *c*-index measure in order to compare the predictive ability of proposed models, being a *c*-index of 1 predictive of a perfect agreement [196]. FIGO stage, hormonal status, histological subtype and differentiation grade are relevant prognostic variables for EOC survival. In this study, the predictive value of hormonal status and tumor stage for EOC risk of death was 0.616 (Model 1), although the model's predictive ability increase to 65.1% with the inclusion of histological subtype variable (Model 2) and to 66.1% with the further inclusion of differentiation grade (Model 3). Concerning the influence of *GSR* rs3779647 polymorphism on the capacity to predict death by EOC at 5-years (Model 4), we observed an improvement on *c*-index after the addition of the genetic information (*c*=0.714). Specifically, in Model 4, we observed a risk of death (at 5 years) 2.07 times higher in patients carrying the C allele than TT genotype (HR, 2.20; 95% CI, 1.07-4.54; *P*=0.032*; and* $P_{bootstrap}$=0.028*).* Lastly, assessing a very stringent model (Model 5), due to the introduction of the platinum sensitivity variable, we noted an enhancement of 26.7% on the capacity to predict death by

EOC when compared with Model 1, obtaining with this model the highest predictive value (*c*=0.883). Still with Model 5, the estimated risk of death was 2.13 times higher for C allele genotype carriers than for TT genotype patients (HR, 2.13; 95% CI,1.04-4.40; *P*=0.040*; and* $P_{bootstrap}$=0.038). Therefore, according to Table 9 and considering the distinct models, the platinum-sensitive phenotype and *GSR* CT/CC genotypes emerged as the most important predictors of EOC death risk at 5-years.

Table 9 - Predictive models regarding the risk of death at 5-years considering different prognostic factors, in the subgroup of patients submitted to incomplete or suboptimal surgical resection.

	HR	95% CI	*P*-value	c-index
Model 1				
FIGO Stage[a]	1.89	0.60-5.20	0.215	0.616
Hormonal status[b]	1.84	1.06-3.17	0.029*	
Model 2				
FIGO Stage[a]	1.78	0.65-4.90	0.265	0.651
Hormonal status[b]	1.85	1.07-3.19	0.028*	
Histological subtype[c]	0.66	0.37-1.15	0.138	
Model 3				
FIGOStage[a]	1.40	0.50-3.93	0.529	0.661
Hormonal status[b]	1.63	0.91-2.92	0.103	
Histological subtype[c]	0.70	0.39-1.29	0.253	
Differentiation Grade[d]	1.83	0.71-4.74	0.212	
Model 4				
FIGO Stage[a]	1.80	0.55-5.94	0.335	0.714
Hormonal status[b]	1.54	0.84-2.83	0.163	
Histological subtype[c]	0.66	0.34-1.29	0.222	
Differentiation Grade[d]	2.17	0.75-6.25	0.151	
GSR[e]	2.20	1.07-4.54	0.032*	
Model 5				
FIGO Stage[a]	0.95	0.28-3.17	0.933	0.883
Hormonal status[b]	1.40	0.75-2.60	0.290	
Histological subtype[c]	0.82	0.41-1.66	0.589	
Differentiation Grade	1.90	0.66-5.46	0.236	
Platinum sensitivity[f]	6.89	3.57-13.28	0.000	
GSR[e]	2.13	1.04-4.40	0.040* †	

*statistically significant value; [a] I/II vs II/IV stages; [b] pre vs post menopause; [c]serous vs others; [d] well differentiated vs other; [e] TT genotype vs C allele: [f] sensitive vs others; † *P*=0.028 and *P*=0.038, respectively, after bootstrap on 1000 samples

- **Secondary outcome: 5-year DFS**

Regarding the 5-year DFS analysis considering the entire cohort, the results did not reveal a significant statistical association for the *PEAR1* rs12041331, *RGS7* rs2502448, *GSR* rs3779647 SNPs, both under log-additive and dominant genetic models (*P*>0.05). Additionally, even when stratifying by FIGO stage and extension of surgical resection, the statistical associations did not reach a significance level for any genetic polymorphism considered, except for GSR rs3779647 polymorphism. Concerning this genetic variant, and

using the log-additive model, we observed significant differences in time to disease recurrence at 5-years between the three genotypes considered. Namely, TT genotype patients showed a prolonged time until disease relapse when compared to CT/CC genotype patients, independently of the FIGO disease stage at diagnosis (*P*=0.047*, and P*=0.013, for early and advanced stages, respectively). Particularly, such association become even more evident within the subgroup of early stage patients for which the cytoreductive surgery was considered incomplete/suboptimal *(P*=0.016). Similar results were found in the dominant model, where we observed significant differences between carriers of C allele and TT genotype (*P*=0.043 and *P*=0.051 for early and advanced stages, respectively).

Specifically, within the subgroup of patients with advanced disease at diagnosis and surgical residual disease higher than 1cm, a prolonged time until disease recurrence was observed for TT genotype patients when compared with C allele patients (*P*=0.039) (Figure 9). Nevertheless, this result was not corroborated in the multivariate analysis (*P*=0.163, Table 10).

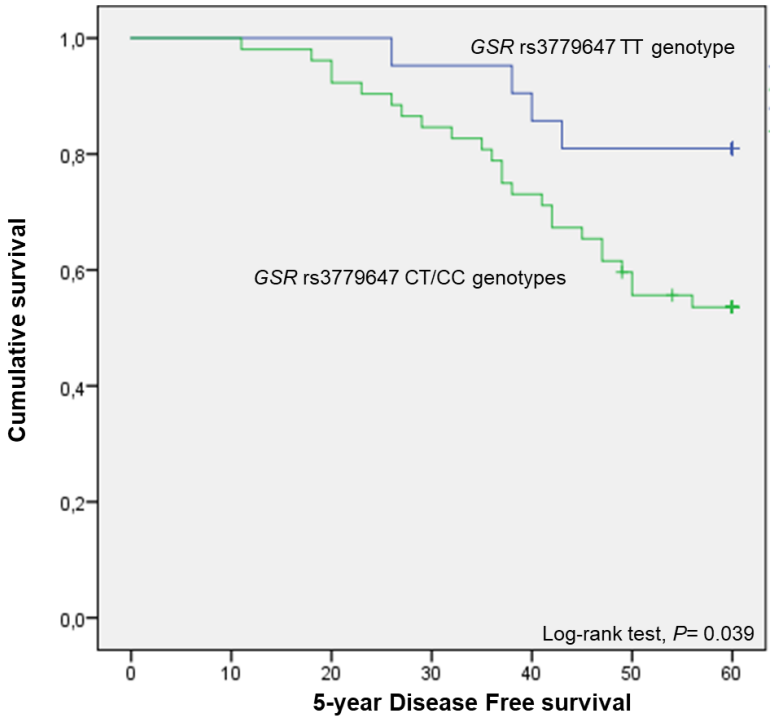

Figure 9 - Disease-free survival at 5-years by Kaplan-Meier and log-rank test for the subgroup of EOC patients with FIGO III/IV stage disease at diagnosis and submitted to incomplete/suboptimal surgical resection, according to *GSR* rs3779647 polymorphism genotypes (dominant genetic model). The group of patients with TT genotype had significantly prolonged time until disease recurrence when compared to C allele carrier patients (*P*=0.039).

Table 10 - Multivariate Cox regression analyses on the risk of death and recurrence at 5-years for the EOC cohort, according to several clinicopathological parameters.

	5 year- Overall survival			5 year- Disease free survival		
	aHR[(a)]	95% CI	*P*-value	aHR[(a)]	95% CI	*P*-value
***PTGS2* rs20417 GG vs GC/CC**	0.82	0.52-1.29	0.391	0.65	0.34-1.23	0.182
FIGO I/II	0.98	0.23-4.08	0.974	1.08	0.20-5.70	0.930
Complete/ optimal surgery	0.63	0.10-4.15	0.633	0.26	0.03-2.66	0.254
Others	0.22	0.00- NE	0.995	1.81	0.00- NE	0.952
FIGO III/IV	0.95	0.59-1.55	0.851	0.68	0.33-1.37	0.275
Complete/ optimal surgery	0.40	0.10-1.64	0.203	0.50	0.12-2.17	0.357
Others	1.21	0.69-2.13	0.504	0.89	0.36-2.22	0.809
***TBXA2R* rs1131882 GG vs AG**	1.34	0.86-2.10	0.199	1.30	0.71-2.40	0.394
FIGO I/II	2.33	0.53-10.18	0.261	3.51	0.67-18.39	0.137
Complete/ optimal surgery	1.08	0.12-9.99	0.944	1.54	0.16-15.15	0.714
Others	NE	NE	NE	NE	NE	NE
FIGO III/IV	1.05	0.65-1.70	0.834	0.95	0.49-1.85	0.878
Complete/ optimal surgery	0.90	0.21-3.84	0.888	1.26	0.28-5.71	0.768
Others	1.40	0.81-2.42	0.234	1.22	0.48-3.11	0.683
***ITGB3* rs5918 TT vs TC/CC**	0.82	0.49-1.35	0.426	0.91	0.47-1.76	0.771
FIGO I/II	NE	NE	NE	NE	NE	NE
Complete/ optimal surgery	NE	NE	NE	NE	NE	NE
Others	NE	NE	NE	NE	NE	NE
FIGO III/IV	0.84	0.50-1.40	0.499	0.94	0.48-1.86	0.861
Complete/ optimal surgery	1.09	0.28-4.28	0.906	1.33	0.34-5.21	0.685
Others	0.82	0.44-1.51	0.518	1.07	0.44-2.63	0.882
***PEAR1* rs12041331 GG vs AG/AA**	1.06	0.63-1.78	0.832	0.97	0.48-1.98	0.937
FIGO I/II	1.62	0.31-8.42	0.568	2.35	0.40-13.75	0.342
Complete/ optimal surgery	1.51	0.16-14.24	0.721	2.29	0.22-23.63	0.486
Others	6.99	0.00- NE	0.993	NE	NE	NE
FIGO III/IV	0.99	0.56-1.73	0.966	0.87	0.39-1.92	0.729
Complete/ optimal surgery	0.67	0.15-2.90	0.589	0.90	0.19-4.29	0.893
Others	1.08	0.55-2.11	0.827	1.12	0.37-3.35	0.846
***RGS7* rs2502448 TT vs TC/CC**	0.86	0.54-1.37	0.523	1.29	0.65-2.58	0.467
FIGO I/II	1.20	0.24-6.02	0.823	2.15	0.25-18.58	0.487
Complete/ optimal surgery	NE	NE	NE	NE	NE	NE
Others	NE	NE	NE	0.16	0.00- NE	0.865
FIGO III/IV	0.90	0.55-1.47	0.659	1.31	0.63-2.74	0.476
Complete/ optimal surgery	0.79	0.24-2.65	0.708	0.91	0.25-3.34	0.892
Others	0.83	0.47-1.46	0.513	1.51	0.50-4.54	0.461
***GSR* rs3779647 TT vs TC/CC**	1.28	0.76-2.14	0.359	1.196	0.60-2.38	0.610
FIGO I/II	0.33	0.06-1.80	0.199	0.13	0.01-1.22	0.073
Complete/ optimal surgery	0.23	0.02-2.36	0.216	0.07	0.00-1.10	0.059
Others	0.14	0.00- NE	0.993	0.02	0.00- NE	0.887
FIGO III/IV	1.78	1.00-3.16	0.050	1.75	0.80-3.86	0.163
Complete/ optimal surgery	0.46	0.13-1.65	0.235	0.69	0.18-2.61	0.581
Others	**2.56**	**1.96-5.49**	**0.016**	3.00	0.86-10.43	0.085

Bold values are statistically significant; (a) Adjusted for hormonal status (pre vs post menopause), histologic subtype (serous vs others), age (> 60 vs <60 years) and differentiation grade (well differentiated vs others); NE- non-estimated

5. Discussion

OC represents one of the leading causes of morbidity and mortality among gynecological cancers, despite its relatively low incidence [4]. The lack of specific and effective screening strategies, the indolent and asymptomatic nature of the disease have been pointed as the key reasons for the high OC-related lethality [185]. Although OC is considered to be a chemosensitive neoplasia, with a high rate response to the platinum-based first-line therapy, the manifestation of a resistant treatment phenotype by most of patients constitutes a major obstacle, which severely affects the 5-year survival rate of these patients (about 45%) [186-189]. Therefore, the optimization of OC first-line therapeutic strategies and the improvement of the balanced and profitable integration of emergent biological agents are fields of intense research that would benefit from the identification of predictive and prognostic biomarkers. It is highly recognized that the inter-individual variability, particularly associated with genetic polymorphisms located in specific genes, might represent helpful predictive and prognostic determinants for these patients [198].

Due to the essential role of ASA not only as an inflammatory inhibitor but also as a chemoprotective agent against OC, the AR manifestation might potentiate the inflammatory process and contribute to the development, clinical course and prognosis of OC patients [55, 161-163]. In that sense, it may be interesting to evaluate the influence of AR-related genetic polymorphisms in the clinical outcome of these patients. To the best of our knowledge, this is the first study that tests the impact of genetic variants described in literature to be associated with AR in the risk of death and relapse of a cohort of EOC patients.

5.1. Association of selected polymorphisms with the clinical outcome of EOC patients

Although the selected genetic polymorphisms have been related to AR phenotype, growing evidences have shown the relevant role of those polymorphisms, or their genes, in the susceptibility and progression of malignant diseases, being some of these determinants specifically studied in ovarian tumors.

5.1.1. SNPs selected from candidate gene studies

The COX-2 enzyme, encoded by the *PTGS2* gene, has an essential role in inflammation and tumorigenesis, being expressed through the influence of cytokines, mitogens and prostaglandins [45, 199]. Particularly, the COX-2 overexpression has been

correlated with growth, invasion, and migration of malignant ovarian cells as well as the acquisition of chemoresistance and angiogenic features, potentially affecting its clinical outcome [200-202]. Namely, several studies have shown that higher COX-2 expression significantly contributes to increased risk of death and relapse among OC patients [203-205]. The non-coding transcript exon variant rs20417, located in the promoter region of *PTGS2* gene, comprises a substitution of Guanine (G) to Cytosine (C) at the -765 position. The alternative allele (C allele) has been associated with a lower promoter activity when compared to the reference allele, which precludes the overexpression of COX-2 and thus preventing a poor prognosis of these patients [206]. Our results showed a prolonged survival associated with CC genotype, which is in accordance with the putative functional effect of this SNP. Nevertheless, none of the associations reached the statistical significance, even when performed a subgroup analysis according to FIGO stage.

The *Integrin beta-3* (*ITGB3*) gene encodes the beta 3 subunit of a receptor protein (GPIIIa), known as integrin aIIbβ3, which is found on the surface of platelets. Higher expression of β3 integrins is a common trait observed in EOC cells, in opposite to normal ovarian cells, being considered that β3 integrins are able to promote survival and proliferation of OC cells [207, 208]. Additionally, the ability for OC cell invasion and migration appear to be strictly dependent on integrin β3 [208, 209]. The missense variant rs5918 present in this gene, leads to the alteration of thymine (T) to cytosine (C) in exon 2, resulting in the substitution of leucine (P1A1) to a proline (P1A2) at amino acid 33 [97, 132]. Some authors refer the relevance of rs5918 SNP in cancer development once this genetic alteration might modulate the activation of intracellular signaling routes as well as increase cell aggregation properties [210-212]. However, although leading to an aminoacid substitution, the functional impact of the rs5918 polymorphism is not yet fully established. For instance, it is also considered that the alternative variant could affect splicing regulation due to its influence in the exon splicing enhancer binding motif activity [213]. Namely, Bojesen *et al*. (2005) propose that rs5918 SNP might influence the cellular adhesive properties, which might be associated with selective advantages that promote the growth and progression of OC cells, including prompting of migration, survival and/or extracellular matrix adhesion [208]. According to our results, patients with *ITGB3* rs5918 TT genotype exhibited a lower 5-year OS rate when compared to CT/CC genotype patients, mainly when considering the subgroup of patients with early disease stage at diagnosis (FIGO I/II, *P*=0.036). These results seem to be inconsonant with the previous evidence as the alternative allele (C allele) has been associated with a more aggressive phenotype and, hence, potentially associated with a poorer prognosis of cancer patients. Nevertheless, this significant impact was not confirmed in the multivariate analysis or even among the

subgroup of patients with FIGO III/IV disease stage at diagnosis. Due to the limited sample size included in the stratified analysis, confirmatory studies are indispensable to prove the functional role of this variant and its clinical relevance in this clinical setting.

The TXA2-receptor interacts with TXA2, being involved with platelet activation/ aggregation as well as hemostasis regulation [144, 149]. Additionally, TXA2 may induce the activation of several signaling cascades, regulating a wide range of cellular processes such as cellular adhesion, growth, motility and survival and even the inflammatory response [214]. The high expression of *TBXA2R* gene has been described in several types of cancer associated with a poor prognosis, being particularly well-studied in lung cancer [214-216]. Regarding OC, the relevance of this gene was not specifically portrayed, however, some studies suggest a key role of TXA2 in OC development, due to its higher expression levels in OC tissues than in normal ovarian ones [217-219]. In addition, it has been demonstrated that OC cells can promote AA-induce platelet activation by mediation of the TXA2-receptor, and the recruitment of platelets by tumor cells has already proven to be essential for their survival and progression [218, 219]. Although the functional role of *TBXA2R* rs1131882 is not yet understood, several hypotheses have been suggested, as we presented in Supplementary Table 1. Based on these assumptions, it is proposed that rs1131882 A allele (variant allele) affects, direct or indirectly, the gene transcription, and hence, increases the levels of TXA2 receptor. Therefore, it is considered that the presence of the alternative allele might favor platelet aggregation and, thus, protect EOC cells from immune surveillance which, ultimately, could negatively affect the clinical outcome of these patients. These evidences appear to be corroborated by our results, as it was observed a significant improved 5-year OS and DFS for *TBX2AR* rs1131882 GG genotype patients compared to AG patients, who were diagnosed at early stages and submitted to incomplete/sub-optimal cytoreduction (P=0.029 for 5-year OS; mean of survival time, 55.6 and 45.50 months, respectively; P=0.002 for 5-year DFS). The fact that significant associations are registered in patients undergoing incomplete surgery is expected since the literature has already shown that the presence of residual disease after cytoreductive surgery is accompanied by high TXA2 levels [217]. Consequently, high TXA2 levels are available to bind to its receptors and, in the presence of rs1131882 T allele, the TXA2 receptors levels are also increased which potentiate platelet aggregation/ inflammatory processes that, ultimately contribute to a poor prognosis of these patients. Increased TXA2 level seems to be more preponderant for patients with less tumoral load when compared to patients with disseminated disease. Inclusively, the establishment of distant metastasis might reflect the expression of a panoply of other pro-inflammatory mediators with a more relevant role than TXA2 in the acquisition and manifestation of an aggressive clinical phenotype. However, these results were not

corroborated by multivariate analysis by the limited number of included patients after stratification, which might influence the achievement of reliable conclusions.

5.1.2. SNPs selected from GWAS

PEAR1 is expressed in platelets and endothelial cells and has been pointed to significantly influence the sustained platelet aggregation through the platelet integrin αIIbβ3 [158]. *PEAR1* has also been recognized as a modifier of neoangiogenesis, contributing to pathologies such as inflammatory disease, retinopathy, and cancer, which show an excessive growth of vessels [220]. Despite the insufficient data suggesting a relationship between *PEAR1* and cancer, it has been speculated the role of the TGF-β signaling system in the development of malignancies associated with *PEAR1* gene. In fact, it was also demonstrated that the *PEAR1* is co-expressed with other genes which are part of TGF-β signalling system (e.g. *ACVRL1* and *RhoJ* genes) and are important in blood vessel formation as well as in proliferation and migration of endothelial cells [221]. Taking into account the functional role of rs12041331 SNP, an intronic variant, it is predictable that the presence of G allele might be associated with a poorer prognosis in EOC patients due to its linkage with higher expression of *PEAR1* [159]. However, in this study, no significant associations were found between rs12041331 and 5-year OS and DFS, independently of genetic model or stratification performed (P>0.05). Although the insignificant results achieved, to the best of our knowledge, this is the first study conducted to study the influence of *PEAR1* genetic variants in EOC patients and there are limited studies that focus on cancer. Nevertheless, inflammation, angiogenesis, and platelet aggregation are three critical points of carcinogenesis that have been related with *PEAR1* gene, and additional research might be useful to explain the possible influence of this mediator in cancer promotion and progression.

RGS proteins play a vital role in regulating signaling cascades generated by G protein coupled-receptors through the accelerated inactivation of G-protein. Changes in RGS proteins have been associated with the development of several common diseases as well as with drug addiction [222, 223]. Still, the literature has reported clear differences in RGS proteins expression among numerous solid and hematological tumors [224-226]. Concerning OC, Hurst *et al*. (2009) evaluated the expression levels of RGS proteins observing that RGS transcripts, specifically RGS6, were expressed at higher levels in OC cell lines than in normal ovarian cells. Furthermore, they showed that RGS proteins are able to critically modulate lysophosphatidic acid signaling in OC cells which represents a crucial

mediator of OC initiation and progression, working as an autocrine activator of cellular growth, migration, and survival of cell as well as leading to the production of pro-angiogenic factors. However, the authors did not report a conclusion about the role of RGS7 protein in the development of OC [224]. In fact, the relevance of this protein in oncology remains unclear, requiring additional studies in this domain. Nevertheless, some studies showed that a major pro-inflammatory cytokine, known as tumor necrosis factor-α (TNF-α), may promote *RGS7* expression [227-229].

The rs2502448 genetic variant, located in the intronic region of *RGS7* gene, encompasses an alteration from thymine (T) to cytosine (C) at 18097 position. Postula *et al*. (2012) indicated that rs2502448 SNP might contribute to platelet reactivity in patients subject to ASA therapy, inducing a suboptimal response to this compound. Although the functional effects of this variant are not clear, it is speculated that *RGS7* rs2502448 SNP might potentially affect receptor function through alternative splicing mechanisms or could be in LD with promoter SNPs (causal variants) and, thereby, influence the expression of the gene [193]. Analyzing the presented results, significant differences in survival at 5-years in the subgroup of early disease stage patients who were submitted to incomplete/suboptimal surgical resection were observed according to the *RGS7* rs2502448 genotypes. Namely, the TT genotype patients presented a significantly decreased survival time when compared to the C allele carriers (P=0.010, mean of survival 37.00 and 57.75 months, respectively). According to the evidence previously described, we hypothesized that the TT genotype might be associated with higher levels of RGS7 protein. As consequence, higher protein levels are available to bind to the receptor and, then, might promote a cascade of events leading to inflammatory processes and platelet aggregation. Therefore, these events might contribute to survival, migration and uncontrolled proliferation of tumor cells as well as might activate lysophosphatidic acid (LPA) signaling pathways, which are crucial for OC initiation and progression. Ultimately, this could significantly affect the clinical outcome of TT genotype EOC patients, as observed with our results (Figure 10). However, it is essential emphasized that after stratification, the size of the sample was limited which do not allow to achieve definitive conclusions. Thereby additional functional and larger studies are warranted both to identify the causal variant and the biological mechanisms underlying our findings.

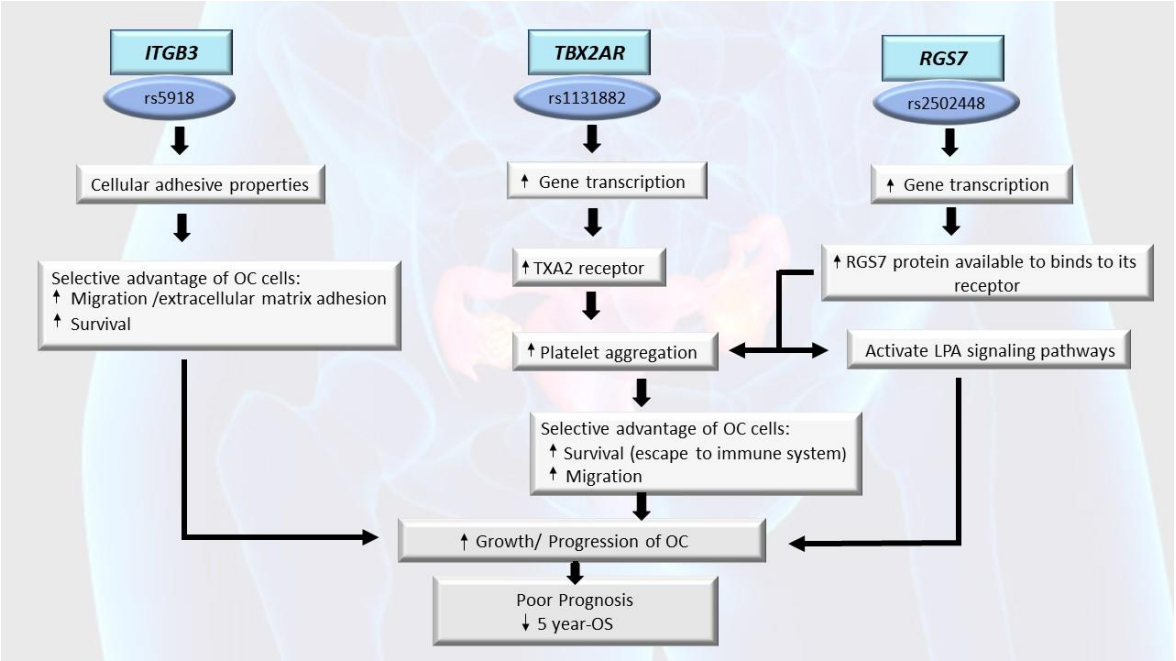

Figure 10 - Potential impact of *ITGB3* rs5918, *TBXA2R* rs1131882 and *RGS7* rs2502448 polymorphisms in OC, according to their putative biological relevance. All SNPs might prompt the acquisition of selective advantage features by OC cells, leading to a poor prognosis of these patients.

The glutathione reductase (GR) appears to have a preventive role in the accumulation of hydroperoxides as well as in the formation of AA metabolites, being considered that GR variability may lead to suboptimal aspirin response [193, 230]. In addition, GR is a crucial enzyme that catalyzes the reduction of oxidized glutathione, being involved in resistance to oxidative stress as well as maintaining high levels of reduced glutathione in the cytosol. The GR is an important element of the antioxidant defense system that protects cells from free radicals, like ROS. Curiously, tumor cells appear to exhibit increased levels of reduced glutathione (GSH) when compared to normal cells [231, 232]. The effect of GSH has been correlated with inflammatory processes, apoptosis evasion, TME aggression and resistance to several drugs. Namely, the resistance to chemotherapeutic drugs appears to be accompanied by high levels of GSH, particularly in the case of platinum containing compounds, anthracyclines, alkylating agents, among others [197, 233]. Namely, platinum-based compounds constitute one of the most active and used cytotoxic agents in the clinical field, particularly for OC treatment [234-237]. Bellote *et al.* (2013) showed that higher levels of GSH in OC cells contribute to platinum-tumor cell resistance despite this evidence appearing to be transversal to other types of tumors [231, 238-243]. In addition, Stordal *et al.* (2012), using OC cell lines, demonstrated that those which were resistant to chemotherapy exhibited overexpression of *GSR* and thus higher levels of reduced glutathione [244].

The conjugation between platinum compounds and GSH may lead to the inactivation of platinum and/or high-water solubility which, in turn, decreases the availability of free drug within the cell to bind to its target and, thus, promoting their excretion from the organism [245]. In that sense, the high levels of GSH available, potentially caused by the elevated expression of GR enzyme, might increase the activity of this detoxification route and, hence, diminishing the pharmacological concentration of platinum-containing compounds within the cell. Lastly, this might limit the platinum-toxic effect in the neoplastic cell, compromising treatment response and thus, significantly affecting patient survival [243, 245, 246].

The intronic variant rs3779647 lies within the promoter flanking region of the *GSR* gene, a member of the class-I pyridine nucleotide-disulfide oxidoreductase, which encodes GR. Regarding the functional effect of this polymorphism, the recent study elaborated by Zhang *et al.* (2019) demonstrated, through immunohistochemical techniques, that the rs3779647 C allele leads to an overexpression of *GSR* in biliary tract cancer cells and, consequently, higher GR levels [247]. According to previously reported assumptions, we hypothesized that the rs3779647 C allele might elevate the *GSR* expression and hence, enhance the GR amount available in EOC cells, predisposing these patients to the poorest prognosis. The results observed in our study corroborated the evidence reported in the literature. In fact, we observed that C allele carriers showed a significantly diminished survival time when compared to the TT genotype patients (P=0.010; mean survival 40.21 and 49.23 months, respectively), particularly in patients diagnosed at advanced stage and submitted to incomplete or suboptimal surgical resection. This may be explained by the fact that patients with advanced disease stage patients submitted to incomplete surgery have a considerable residual disease that needs to be subsequently subject to the action of adjuvant chemotherapeutic agents as platinum-based compounds. Therefore, these cells may exhibit a suboptimal response to this agent, possibly due to the high levels of intracellular GSH available. Moreover, within platinum-sensitive patients, those carrying the rs3779647 C allele exhibited a significantly reduced survival time than TT genotype patients (P=0.036). Furthermore, exploring the role of *GSR* rs3779647 in clinical outcome of EOC patients, we observed that patients diagnosed at advanced stages with residual surgical disease higher than 1cm, and carriers of TT genotype showed a prolonged time until disease recurrence when compared with C allele patients (P=0.039), despite this result was not confirmed in the multivariate Cox analysis.

As previously suggested, the significant results appear to be concordant with a higher detoxification route activity associated with GSH, potentially due to overexpression of *GSR* which might favor a faster platinum excretion and, hence, its reduced cytotoxic effect. Ultimately, it might have an impact on the sensitivity to chemotherapy, and consequently influence the clinical outcome of EOC patients, as previously reported [246].

To strengthen the obtained results, the impact of clinical variables in the risk of death at 5-years was assessed, analyzing the predictive ability of proposed models, among patients submitted to incomplete/suboptimal surgical resection. Higher predictive ability in the estimation of 5-year risk of death was observed in models that encompass the *GSR* polymorphism, i.e, models 4 and 5 (*c*=0.714 and *c*=0.883, respectively). Particularly, in the most rigorous model (model 5), platinum insensitive phenotype (HR, 6.89; 95% CI, 3.57-13.28; *P*<0.001) and *GSR* CT/CC genotypes (HR, 2.13 ;95% CI,1.04-4.40; *P*=0.040*; and* $P_{bootstrap}$=0.038) emerged as the most relevant predictors of EOC death risk at 5-years (Figure 11). Hence, the establishment of a predictive genetic profile that encompasses the *GSR* polymorphism data might be helpful as a biomarker to predict clinical outcome. To the best of our knowledge, this is the first study that assesses the influence of adding *GSR* rs3779647 to numerous EOC clinical variables in the estimation of the risk of death at 5-year and shows the advantageous predictive capability in combination of clinical plus genetic information. Being the present study pioneering in this field, we highlighted the need for further studies that replicate this predictive model in a distinct cohort of EOC patients subject to platinum-based chemotherapy.

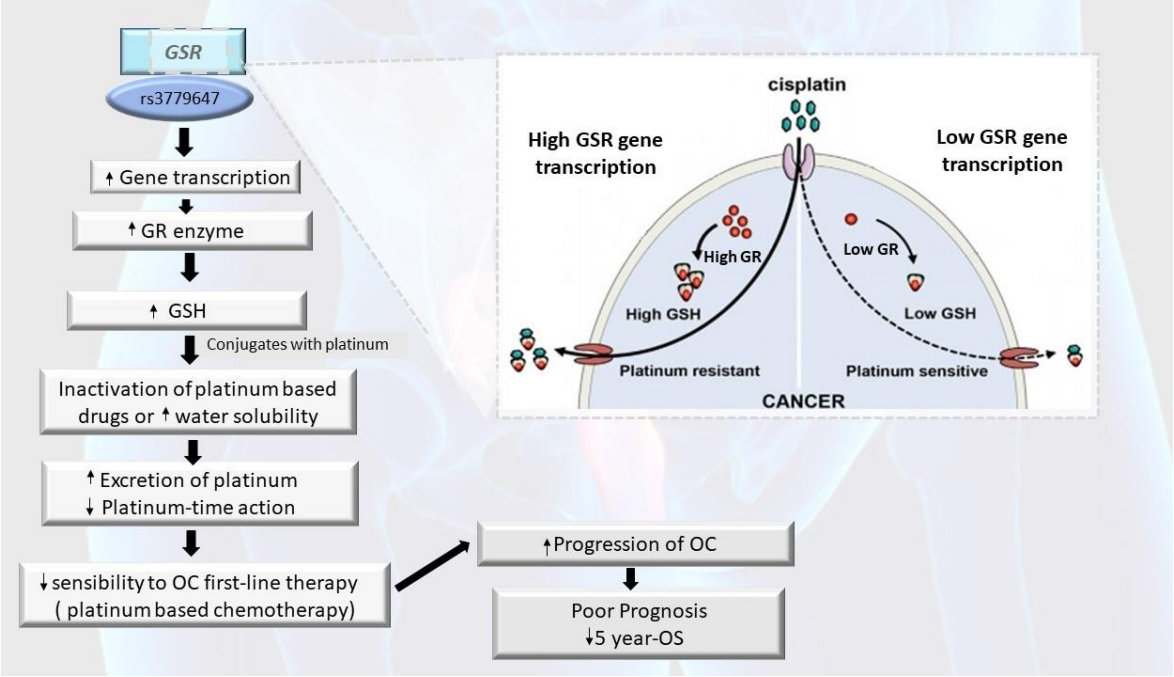

Figure 11 - Potential biological pathways affected by *GSR* rs3779647 variant that might impair a proper action of platinum agents, due to the high platinum excretion rate, and consequent, platinum detoxification from the body which might contribute to a poor EOC prognosis.

In summary, among the six AR-genetic variants selected, four of them showed a significant impact in the clinical outcome for the presented cohort of EOC patients, namely *ITGB3* rs5918, *TBX2AR* rs1131882, *RGS7* rs2502448, and *GSR* rs3779647. Nevertheless, for the *ITGB3* rs5918, *TBX2AR* rs1131882, *RGS7* rs2502448, the results were not reinforced in the multivariate analysis. Despite the present study include a considerable sample size (n=336), when stratifying by FIGO stage and/or extension of surgical resection, the sample size is effectively reduced which undermines the study power and impaired proper and further statistical analyses. For this reason, it is crucial to perform large and well-designed studies to validate our findings. Furthermore, additional studies are urgently required to attest the role of selected genes/functional effect of respective SNPs and its relevance on the prognosis of EOC patients.

Although the studied SNPs were selected based on specific criteria, as the MAF, the allelic frequency observed in this study was lower than the expected for some variants. Namely, regarding TBXA2R rs1131882, only GG/AG genotype patients were observed meaning that no AA genotype patients were registered.

The highlight should be considered for *GSR* rs3779647 SNP that arises as the most promising and consistent result found in the present analysis. The influence of the rs3779647 C allele was significantly observed in the subgroup of advanced stage patients submitted to incomplete or suboptimal surgery, the subgroup that represents a major concern to the oncological gynecology. This variant appears to be involved with insensitivity to platinum-based first-line chemotherapy, a clinical manifestation that is commonly observed among approximately 75% of advanced stage patients. From the predictive analysis performed, *GSR* rs3779647 polymorphism emerged as the most relevant predictor of EOC death risk at 5-years, being a potential biomarker to evaluate clinical outcome. Nevertheless, the magnitude of findings needs to be estimated and confirmed in another OC cohort.

6. Conclusions & Future Perspectives

The present study was performed with the aim to evaluate the impact of six AR-related genetic variants selected from genetic association studies, namely GWAS and candidate gene studies, in the clinical outcome of a Caucasian EOC cohort. To the best of our knowledge, despite showing in the literature that some of the selected SNPs and/or respective genes are associated both with the development of AR and OC, none study directly relates these two entities.

According to our results, four of the six considered polymorphisms, namely *ITGB3* rs5918, *TBX2AR* rs1131882, *RGS7* rs2502448 and *GSR* rs3779647 show a significant impact in the EOC clinical outcome. Nevertheless, the rs5918, rs1131882 and rs2502448 SNPs were not consistently supported in the multivariate analysis, particularly due to the under-power associated with FIGO stratification, namely within the FIGO stage I/II subgroup, which implicates a careful interpretation of these results. Aside from this, the significant associations involving *GSR* rs3779647 variant arise as the most promising and consist finding of this study, being corroborated in the predictive models' analysis. In fact, the present study suggests that this polymorphism might be a helpful biomarker to predict the 5-year risk of death among patients submitted to incomplete or sub-optimal primary cytoreductive surgery. In this field, most of the research has been developed focusing on genetic variants present in genes associated with the expression of glutathione-S-transferase (GST), that also appear to significantly affect platinum-based chemotherapy response and, hence, OC survival [248, 249]. Therefore, further research based on the glutathione-associated genes might be useful to state a genetic profile associated to platinum-based chemotherapy response which in turn, allows the identification of OC predictive subgroups. In fact, it will be preponderant the identification of those patients who will probably benefit from treatment, with the possibility to adjust of therapeutic dosage and the strategies of follow-up.

Nevertheless, the present results are not enough to undoubtedly prove the relevance of AR-associated genetic variants in the prognosis of EOC patients, although they are a promising start. However, in an attempt to effectively comprise the potential linkage between these two entities, it will be required to overwhelm the limitations underlying the AR-phenotype, i.e., to establish standard ad rigorous criteria to accurately define AR and its adequately detection and treatment. Therefore, it will be essential to confirm the contribution of genetic factors, particularly the presented variants in the AR setting (Figure 12) and, as such, it is essential to perform additional large scale, randomized and multicentric genetic association studies mostly using CVD patients. In that sense, and particularly with the interest to validate these AR-associated genetic variants in a Portuguese population, a parallel study is being performed recurring to a cohort of patients diagnosed with stroke who are subject to therapy with ASA. Moreover, the putative

functional effect of presented genetic variants should be evaluated and, therefore, further analyses should be conducted to fine map regions where SNPs are located, in attempt to confirm if the particular polymorphism is the causal variant or is in LD with it, as well as to the accomplishment of functional studies that evaluate the regulation of gene expression. Finally, the clinical relevance of selected genetic polymorphisms should be addressed, specifically as predictive and prognostic biomarkers in ovarian malignancies. In that sense, it is important to perform large and well-designed studies in independent EOC cohorts that reinforce our findings. Furthermore, these studies should also take into consideration if patients are being submitted to ASA therapy or other anti-inflammatory drugs and identify those who develop resistance or suboptimal responses.

The trend in literature seems to confirm the relevance of aspirin as OC adjuvant therapy and future clinical studies might have in consideration the influence of the genetic background to optimize treatment strategies, in the scope of personalized medicine.

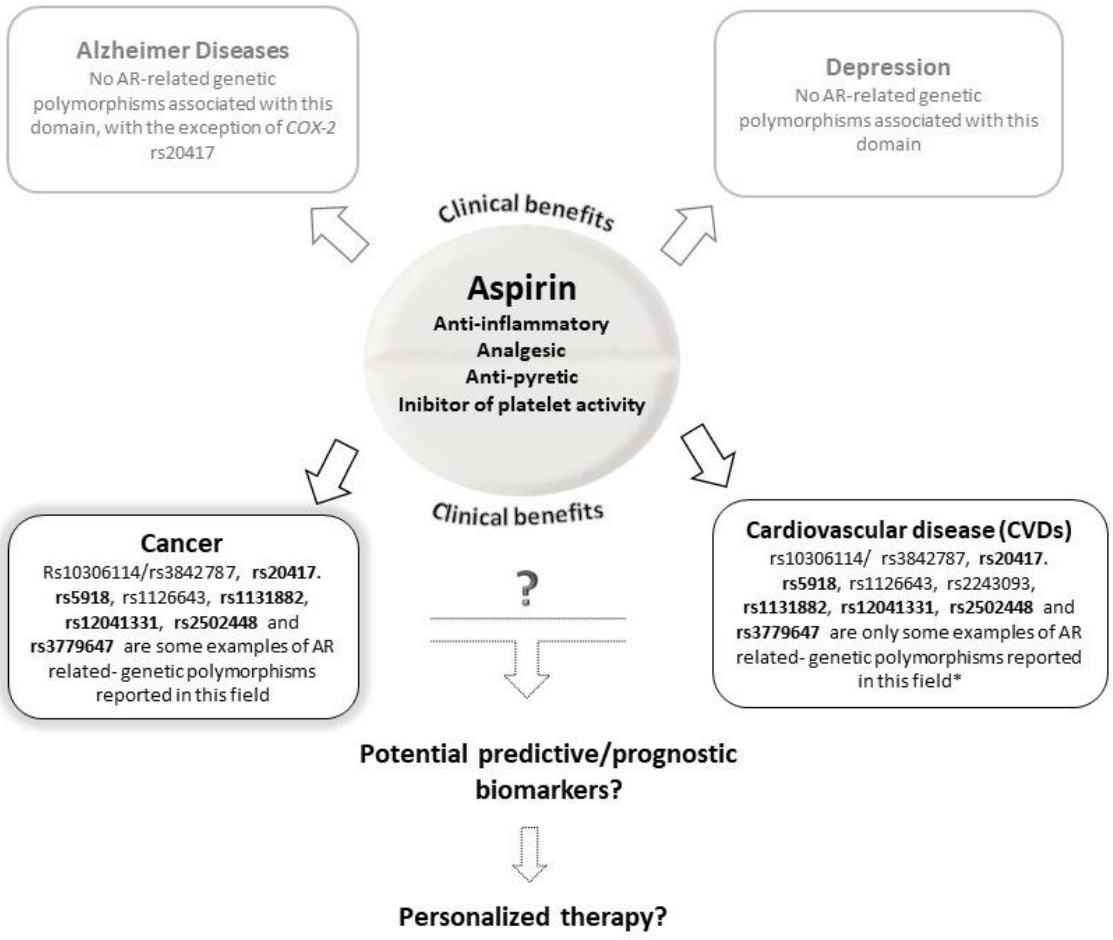

Figure 12 - Clinical benefits of aspirin for several pathologies. Within each pathological group are reported AR-related genetic polymorphisms that are considered in the literature as associated with the respective clinical settings. Whether the relevance of these genetic variants might be validated in these domains, they may be used in clinical practice as potential predictive and prognostic biomarkers and, thus, promoting the personalized therapy development (*AR-related genetic polymorphisms are mainly reported in patients with CVDs).

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8. Appendix

8.1. Appendix 1

Supplementary Table 1 - Genetic polymorphisms reported to affect the response to aspirin

Gene	Gene product	Biological Function	refSNP (rs number)	Variation/ Aliases	MAF[1] EUR	MAF[1] IBS	SNP location	Putative functional effect	Genetic association study [REF]
PTGS1	COX-1	COX 1 is involved in the regulation of AA-induced platelet aggregation and TX production	rs10306114*	A-842G	0.070	0.061	Intergenic (near *PTGS1*)	†	Candidate gene [123, 124]
			rs3842787*	C50T	0.070	0.061	Missense	†	Candidate gene [123, 124]
			rs1236913	C22T	0.056	0.070	Missense	†	Candidate gene[123, 124]
			rs1330344	C-1676T	0.204	0.238	Intergenic (near *PTGS1*)	†	Candidate gene [250]
			*rs10306114 and rs3842787 are in complete LD (r^2=1.0)						
PTGS2	COX-2	COX-2 is involved in the conversion of AA into TXA2, which affect platelet aggregation. This enzyme is only activated under inflammatory states	rs20417	G-765C	0.151	0.150	NC transcript exon	C allele is associated with a lower transcriptional activity of COX-2	Candidate gene [129, 130]
			rs689466	A-1195G	0.187	0.150	Regulatory region variant	A allele is associated with a higher transcriptional activity of COX-2	Candidate gene [250]
ITGB3	GPIIIa	GP IIb/IIIa platelet receptors are specific to fibrinogen, having a role in platelet aggregation and adhesion	rs5918	T1565C (PlA1/A2	0.132	0.136	Missense	It leads to de alteration from leucine to proline in aa sequence. Considered to affect splicing regulation by decreasing the exon splicing enhancer binding motif in the coding sequence containing the same protein domain	Candidate gene [43]
ITGA2	GP Ia	GP Ia/IIa promote the initial interaction between collagen and platelets, regulating the adhesion of platelets and other cell types to the extracellular matrix	rs1126643	C807T	0.403	0.383	Synonymous	T allele is associated with a higher expression of platelet GPIa/IIa receptors.	Candidate gene [130]
GP6	GP VI	GPVI receptor is involved in collagen-induced platelet aggregation and thrombus formation	rs1613662*	T13254C	0.145	0.126	Missense	T allele may be associated with changes in the orientation of some domains of GPVI and may potentially influence GPVI structure/function relationships.	Candidate gene/GWAS [124, 142]
			rs1671152*	A968C	0.141	0.112	Missense	C allele may be associated with changes in the orientation of some domains of GPVI and may potentially influence	Candidate gene/GWAS [142, 151]

Gene	Gene product	Biological Function	refSNP (rs number)	Variation/ Aliases	MAF[1] EUR	MAF[1] IBS	SNP location	Putative functional effect	Genetic association study [REF]
								GPVI structure–function relationships.	
			*rs1613662 and rs1671152 are in complete LD (r^2=1.0)						
GP1BA	GPIb α	GPIb receptor is involved in the formation of platelet plugs by the binding to the A1 domain of vWF, which is already bound to the subendothelium	rs2243093	C-5T Kozak	0.125	0.121	Splice region variant	C allele is associated with a higher expression of the *GP1BA*	Candidate gene [250]
			rs6065	C1018T	0.087	0.093	Missense	T allele may cause a conformational variation in the structure of GPIbα that may affect the ligand binding, once it is closely located to the VWF- and to the thrombin-binding sites	Candidate gene/GWAS [146, 147]
TBXA2R	TXA_2 receptor	TXA_2 receptor interacts with thromboxane A2, being involved with platelet aggregation and with the regulation of hemostasis	rs4523	C924T	0.345	0.322	Missense	T allele may be associated with mRNA stabilization and translation efficiency, or it may be in LD in a mutation associated with the function adjacent to this SNP	Candidate gene [146]
			rs1131882	G795A	0.147	0.173	Missense	A allele may affect the transcription and/or translation efficiency of both isoforms of the TBXA2R gene. This SNP might be in LD with one or several SNPs in the gene promoter region, intronic silencer or enhancer region.	Candidate gene [150]
P2RY1	$P2Y_1$	$P2Y_1$ belongs to the family of G-protein coupled receptors, being involved with platelet shape and aggregation upon ADP binding	rs1065776	C893T	0.050	0.042	Synonymous	Although being a silent alteration, this SNP may lead to changes in ribonucleic acid structure and protein expression. Might be in high LD with the actual causal variant	Candidate gene [154]
			rs701265	A1622G	0.163	0.159	Synonymous	†	Candidate gene [154]
			rs1371097*	C-1382T	0.160	0.159	Regulatory region variant	†	Candidate gene [154]
			rs1439010*	A>G	0.160	0.159	Intergenic	†	Candidate gene [154]
			rs2312265*	A>G	0.163	0.159	Intergenic	†	Candidate gene [154]
			rs1371097, rs1439010 and rs2312265 are in complete LD (r^2=1.0)						

Gene	Gene product	Biological Function	refSNP (rs number)	Variation/ Aliases	MAF[1] EUR	MAF[1] IBS	SNP location	Putative functional effect	Genetic association study [REF]
P2RY12	$P2Y_{12}$	$P2Y_{12}$ belongs to the family of G-protein coupled receptors, being involved with platelet shape and aggregation upon ADP binding	rs9859538	C16582T	0.444	0.477	Intronic	†	Candidate gene [154]
			rs1491974	T5093A	0.484	0.453	Intronic	†	Candidate gene [154]
			rs10513398	A>G	0.480	0.453	Intronic	†	Candidate gene [154]
			rs3732765	G3629A	0.381	0.374	Missense	†	Candidate gene [154]
			rs10935838*	C139T	0.173	0.159	Intronic	†	Candidate gene [136, 251]
			rs2046934*	T344C	0.173	0.159	Intronic	†	Candidate gene [136, 251]
			rs6809699*	G52T	0.171	0.154	Synonymous	†	Candidate gene [136, 251]
			* rs10935838, rs2046934 and rs6809699 are in complete LD (r^2=1.0)						
PEAR1	Platelet endothelial aggregation receptor-1	The receptor encoded are involved in megakaryopoiesis and platelet activation	rs12041331*	A>G	0.092	0.145	Intronic	G allele is associated with an increased PEAR1 expression as well as platelet PEAR1 protein content	Candidate gene/GWAS [142, 159]
			rs12566888*	G>T	0.092	0.145	Intronic	T allele is associated with an increased PEAR1 expression	Candidate gene/GWAS [142, 159]
			rs2768759	A>G	0.280	0.327	Intergenic (near *PEAR1* and *NTK1*)	†	Candidate gene [252]
			* rs12041331 and rs12566888 are in LD (r^2=0.85)						
PLA2G7	Platelet-activating factor acetylhydrolase	The enzyme modulates the action of platelet-activating factor (PAF)	rs7756935	T35488C	0.244	0.294	Intronic	†	Candidate gene [150]
F13A1	Coagulation Factor XIII	The coagulation factor XIII is involved in the blood coagulation cascade	rs5985	G34T	0.242	0.238	Missense	†	Candidate gene [136]
ADRA2A	alpha-2 adrenoreceptor	The receptor encoded belongs to the G protein-coupled receptor superfamily, being involved with platelet activity	rs4311994	C>T	0.147	0.178	Intergenic (near *ADRA2A*)	This SNP may be in LD with the causal variant(s) or may represent its long-range regulatory elements	Candidate gene/GWAS [151, 193, 253]
RGS7	Regulator of G-protein signaling 7	Regulates G protein-coupled receptor signaling cascades	rs2502448	T18097C	0.401	0.379	Intronic	The SNP may potentially affect receptor function through alternative splicing mechanisms. Also, it may be in LD with promoter SNPs which have not yet been identified or the SNP.	Candidate gene/GWAS [193, 194]
GSR	Glutathione reductase	Reduces oxidized glutathione in the cytosol, being also involved in preventing the accumulation of hydroperoxides and plays a role in the AA metabolites formation	rs3779647	C-130T	0.428	0.467	Intronic	C allele may be associated with increased glutathione circulation levels	Candidate gene/GWAS [193, 194]

Gene	Gene product	Biological Function	refSNP (rs number)	Variation/ Aliases	MAF[1] EUR	MAF[1] IBS	SNP location	Putative functional effect	Genetic association study [REF]
DPP6	Dipeptidyl Peptidase like protein 6	The protein encoded binds specific voltage-gated potassium channels and alters their expression and biophysical properties	rs1387180	A>G	0.257	0.243	Intronic	†	Candidate gene/GWAS [193, 194]
9p21.3 region	X	X	rs10120688*	A>G	0.499	0.523	Intronic	†	Candidate gene/GWAS [151, 254]
			rs10965219*	A>G	0.499	0.514	Intronic	†	Candidate gene/GWAS [151, 254]
			*rs10120688 and rs10965219 are in LD (r^2=0.97)						

Abbreviations: aa, amino acid; COX, cyclooxygenase; EUR, European; GP, glycoprotein; IBS, Iberian; LD, Linkage Disequilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism, TX, thromboxane, TXA2, thromboxane A2, NC, non-coding

[1]Data obtained from Ensemble Database; † Data not available, X Data not applicable

8.2. Appendix 2

A paper entitled “The emergent phenomenon of aspirin resistance: insights from genetic association studies” has been submitted and accepted for publication in the scientific journal *Pharmacogenomics.*