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Promoter methylation of DNA repair genes as biomarker of response to platinum-based chemotherapy: a pan-cancer investigation

Saulė Gumauskaitė

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Faculdade de Ciências da Universidade do Porto e ao

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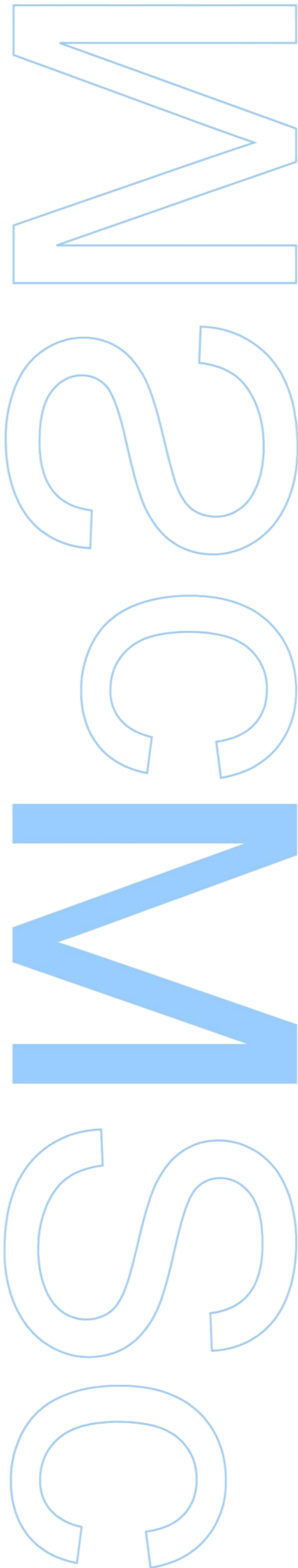

Promoter methylation of DNA repair genes as biomarker of response to platinum-based chemotherapy: a pan-cancer investigation

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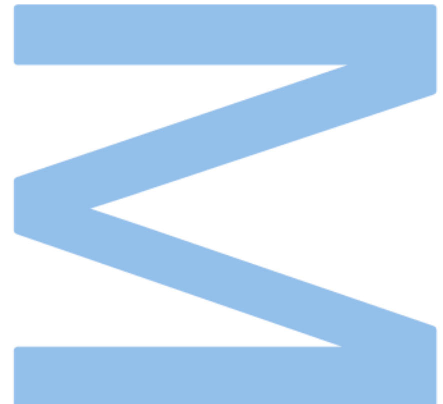

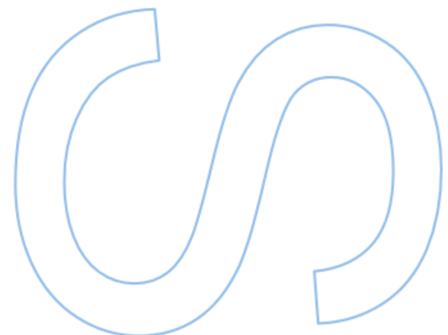

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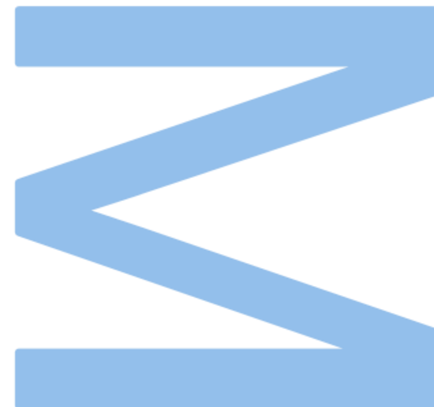

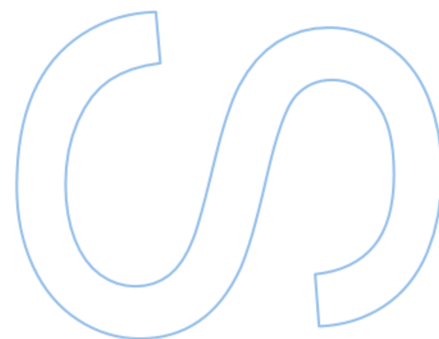

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Sworn Statement

I, Saulė Gumauskaitė, enrolled in the Masters Degree of Biochemistry at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Saulė Gumauskaitė

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Resumo

Introdução: Os fármacos à base de platina (cisplatina, carboplatina e oxaliplatina) são os agentes de quimioterapia mais utilizados no tratamento de diversos tumores sólidos e hematológicos. No entanto, existem diversos desafios no que respeita à sua utilização, nomeadamente no que concerne aos seus severos efeitos secundários e à resistência intrínseca ou adquirida à quimioterapia. Esta última problemática poderia ser reduzida, no caso de ser possível prever a resistência à quimioterapia utilizando biomarcadores de predição de resposta. Os genes de reparação de DNA estão intimamente envolvidos no processo biológico de reparação de danos causados pelos platinos no DNA, sendo a sua expressão frequentemente regulada pela metilação do DNA, podendo assim servir como biomarcadores epigenéticos. A aplicação de biomarcadores epigenéticos tem elevada utilidade clínica, pois estes podem ser detetados em doentes com a utilização de biópsias líquidas não-invasivas, e indicar com elevada acuidade a predição de resistência dos doentes à quimioterapia baseada em platinos.

Métodos: Selecionaram-se cinco genes de reparação do DNA (*BRCA1*, *APEX2*, *RAD51C*, *RBBP8*, *ERCC8*) com base numa análise *in silico* para tumores do esófago (CE), gástricos (CG), coloretais (CRC), da bexiga (CB), do pulmão (CP) e de células germinativas do testículo (TCGT). Identificaram-se os dinucleótidos CpG relevantes para o desenho de primers e sondas para qMSP, otimizaram-se os parâmetros do qMSP e foram criados dois painéis de genes de reparação de DNA. Após a seleção dos doentes para o estudo, foram utilizadas lâminas de tecidos incluídos em parafina para extração de DNA e posterior tratamento de modificação bissulfito. Finalmente, todas as amostras foram avaliadas por qMSP, e os resultados obtidos foram analisados com software de análise estatística.

Resultados: Os resultados obtidos nas amostras pré-quimioterapia indicam diferenças estatisticamente significativas quando se comparam doentes não responsivos à quimioterapia (e recidivas em CB e TCGT) com doentes que responderam parcialmente/totalmente (que não recidivaram em BC e TCGT). Para além disso, o *BRCA1* apresentou níveis de metilação significativamente diferente entre as amostras pré- e pós-quimioterapia, sendo independente da resposta ao tratamento ou do platino utilizado. Não se observaram diferenças significativas para os restantes genes de reparação do DNA estudados (*APEX2*, *RAD51C*, *RBBP8*, *ERCC8*).

Conclusões: Assim, a metilação do promotor do *BRCA1* é um candidato promissor a biomarcador epigenético útil para prever a resposta dos doentes a agentes

de quimioterapia à base de platina antes do tratamento. Além disso, as dinâmicas da metilação do *BRCA1* em amostras pré- e pós-quimioterapia podem indicar o desenvolvimento de resistência adquirida à terapia, podendo contribuir para estabelecer um alvo promissor para a aplicação de fármacos epigenéticos.

Palavras-chave: quimioterapia à base de platinos; resistência; genes de reparação de DNA; biomarcadores epigenéticos; metilação; qMSP.

Abstract

Background: Platinum-based drugs (cisplatin [CDDP], carboplatin, oxaliplatin) are widely applied chemotherapy drugs to treat various solid cancers and haematolymphoid malignancies. However, several challenges regarding the successful treatment application exist such as severe treatment side effects and intrinsic or acquired resistance to the chemotherapy, which could be reduced in case it was possible to foresee the resistance using predictive biomarkers. DNA repair genes are closely involved in the process of fixing platinum-caused DNA damage and their expression is frequently regulated by methylation in neoplastic cells thus they could serve as epigenetic biomarkers. The application of epigenetic biomarkers has high clinical utility since they can be assessed non-invasively in patient body liquids and predict resistance to platinum chemotherapy.

Methods: Five DNA repair genes were chosen (*BRCA1*, *APEX2*, *RAD51C*, *RBBP8*, *ERCC8*) with preliminary *in silico* analysis for esophageal (EC), stomach (SC), colorectal (CRC), bladder (BC), lung (LC) cancers and testicular germ cell tumor (TGCT). Relevant CpG sites were pinpointed for the construction of quantitative methylation-specific PCR (qMSP) primers and probes. qMSP parameters were optimised and two DNA repair gene panels were established. After patient selection, tumor tissue FFPE slides were used for DNA purification, bisulfite treatment. Finally, all the samples were run with qMSP, and the results obtained were analysed with statistical analysis software.

Results: Pre-chemotherapy samples showed statistically significant differences in *BRCA1* methylation levels between poor (relapsers in BC, TGCT) and partial/complete responders (non-relapsers in BC, TGCT). In addition, *BRCA1* presented significantly different methylation levels in pre- and post-chemotherapy samples, independently of treatment response and type of platinum drug. No significant differences were obtained for the remaining DNA repair genes (*APEX2*, *RAD51C*, *RBBP8*, *ERCC8*).

Conclusions: *BRCA1* is a promising candidate for an epigenetic biomarker that may predict patients' response to platinum. In addition, the dynamics of $BRCA1_{me}$ in pre- and post-chemotherapy samples, suggest that this biomarker may indicate the emergence of acquired resistance and might be a target for epigenetic drug application.

Keywords: platinum chemotherapy; resistance; DNA repair genes; epigenetic biomarkers; methylation; qMSP.

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chemotherapy. Red horizontal lines represent median methylation levels, n indicates the number of patient samples. 43

Abbreviations

5-aza	5-azacytidine
5-FU	5-fluorouracil
5mC	5-methylcytosine
ABCB1	ATP binding cassette subfamily B member 1
AJCC	American Joint Comitee on Cancer
APC	Adenomatous polyposis coli
APE2	Nuclease encoded by *APEX2* gene
APEX2	Apurinic/apyrimidinic endodeoxyribonuclease 2
$APEX2_{me}$	Methylated apurinic/apyrimidinic endodeoxyribonuclease 2
BC	Bladder cancer
BER	Base excision repair
BMP4	Bone morphogenetic protein 4
BRCA1	Breast cancer type 1
$BRCA1_{me}$	Methylated breast cancer type 1
BRD9	Bromodomain-containing protein 9
CAP	College of American Pathologists
Casp8AP2	Caspase 8 associated protein 2
CC	Cervical cancer
CDDP	Cis-diamminedichloroplatinum (II)
CFLAR	CASP8 and FADD-like apoptosis regulator
CG	Cytosine followed by guanine
COL1A1	Collagen type I alpha 1 chain
CpG	Cytosine-phosphate-guanine
CRC	Colorectal cancer
ctDNA	Circulating tumor DNA
CTR1, *CTR2*	High affinity copper uptake protein 1 and 2
ddPCR	Droplet digital PCR
DSB	Double strand break
DNMT1	DNA methyltransferase 1
DNMT3A, *DNMT3B*	DNA methyltransferase 3A and 3B
EC	Esophageal cancer
ECAD	Cadherin 1
ERCC1, *ERCC8*	Excision repair cross complementation group 1 and 8
F+R	Forward and reverse

FANCF	Fanconi anemia complementation group F
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
FGF5	Fibroblast growth factor 5
FHIT	Fragile Histidine Triad Diadenosine Triphosphatase
GC	Gastric cancer
GDA	Guanine deaminase
GULP1	GULP PTB domain containing engulfment adaptor 1
H&E	Haematoxylin and eosin
HIC1	HIC ZBTB Transcriptional Repressor 1
HMG	High mobility group
hMSH2	Human mutS homolog 2
HNC	Head and neck cancer
HOXA9	Homebox A9
HR	Homologous recombination
ICAM1	Intercellular Adhesion Molecule 1
IGFBP-3	Insulin Like Growth Factor Binding Protein 3
ITH	Intratumor heterogeneity
LC	Lung cancer
LOXL1	Lysyl Oxidase Like 1
LRP12	LDL Receptor Related Protein 12
MG98	A type of antisense oligonucleotide
MGMT	O-6-methylguanine-DNA methyltransferase
MLH1	MutL homolog 1
MM	Multiple myeloma
MMR	Mismatch repair
MSP	Methylation-sensitive PCR
MT1E	Metallothionein 1E
NCI	National Cancer Institute
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NSCLC	Non-small-cell lung cancer
NPV	Negative predictive value

OC	Ovarian cancer
p73	Protein related to the p53 tumor protein
PARP	Poly (ADP-ribose) polymerase
PAX5	Paired box 5
PCR	Polymerase chain reaction
PPV	Positive predictive value
PTM	Posttranslational modifications
qMSP	Quantitative methylation-specific PCR
RAD51C	RAD51 paralog C
$RAD51C_{me}$	Methylated RAD51 paralog C
RARB	Retinoic Acid Receptor Beta
RASSF1A	Ras association domain family 1 isoform A
RBBP8	RB binding protein 8
$RBBP8_{me}$	Methylated RB binding protein 8
RG108	N-Phthalyl-L-tryptophan
RIP3	Receptor-interacting protein kinase 3
RT	Room temperature
S100P	S100 Calcium Binding Protein P
SC	Stomach cancer
SCLC	Small-cell lung cancer
SLFN11	Schlafen Family Member 11
SMART	Shiny Methylation Analysis Resource Tool
TCGA	The Cancer Genome Atlas
TFAP2E	Transcription Factor AP-2 Epsilon
TGCT	Testicular germ cell tumor
TIMP4	TIMP Metallopeptidase Inhibitor 4
TLX3	T Cell Leukemia Homeobox 3
TRG	Tumor regression grading
TSG	Tumor suppressor gene
UC	Urothelial cancer
UCSC	University of California, Santa Cruz
WHO	World Health Organisation
WISP2	WNT1-inducible-signaling pathway protein 2

1. INTRODUCTION

1.1. Platinum-based chemotherapy

Platinum-based agents (cisplatin (CDDP), carboplatin, and oxaliplatin) are commonly used for chemotherapy treatment in several types of cancer [1]. The application of these drugs is such prevalent that approximately 50% of all patients who undergo chemotherapy treatment receive one of the platinum drugs [2]. In the spectrum of antineoplastic effects caused by platinum agents are mainly DNA lesions, which actively demonstrate their role as alkylating agents. Eventually, the DNA damage causes antiproliferative arrest, known as the phenomenon of senescence [3], or mitochondrial pathway of apoptosis [4]. An increasing amount of knowledge emphasizing the mechanism of platinum-agent action indicates that the drugs not only act in the cell nucleus but also the cytoplasm [5]. Even though the mechanism of action of the alkylating agents is not fully comprehended, they are widely employed in monotherapy or combined with other agents due to high rates of clinical responses. Several challenges in the use of platinum agents like drug resistance, which will be discussed later, require investigation to better understand the mechanism of action and design of improved treatment strategies or even new platinum-containing chemotherapeutics.

1.1.1. Application

Platinum anticancer drugs are routinely used in the treatment of several types of neoplasias nowadays, mostly solid but also liquid ones, like hematolymphoid malignancies. CDDP anti-tumoral activity was discovered in 1969 and was approved by the United States Food and Drug Administration (FDA) in 1978. Starting then platinum-based chemotherapy treatment emerged, which demonstrates the need for the drug to be a subject of extensive study to discover its mechanism of action and resistance on the molecular level, substantial for contemporary oncology [6, 7]. Alkylating agents have proven effective in the treatment of such distinct models as esophageal (EC), gastric (GC), lung (LC) (small-cell [SCLC] and non-small-cell [NSCLC]), colorectal (CRC), and head and neck (HNC) cancer [8]. It is also used in urothelial (UC), cervical (CC) carcinomas, as well as, testicular and ovarian germ cell tumors (TGCT, OC). Platinum-based compounds are of wide use which can be illustrated by their successful applications to treat other malignancies like leukemias, melanoma, neuroendocrine neoplasms, sarcomas, and tumors of neuroectodermal origin (e.g., neuroblastoma) [9].

The possible theory behind this is that such utility in a wide spectrum of tumor types (entailing different biology, genomic drivers, risk factors, and molecular backgrounds) could emerge from multiple pathways in which platinum-based drugs interfere.

TGCT is an outstanding example of successful treatment with platinum compounds, especially using CDDP, demonstrating high cure rates (~80%) even in cases with metastatic disease [10-12]. It is important to shed light on this mechanism where it is the specific type of testicular tumor that shows hypersensitivity to CDDP which is known to be related to its epigenetic and developmental background and clinicians have not been successful yet to reproduce such treatment rates on somatic-type tumors of adulthood treated with the same platinum-based compounds [13-15].

It has been already years since CDDP was applied as a first-line treatment, both alone or in combination with other therapeutic options, such as radiation (serving as a radiosensitizer) or with other chemotherapeutics [16]. It can be administered in neoadjuvant (for tumor shrinkage) or adjuvant (to lessen the risk of recurrence) therapy settings [17-19]. It also can be employed in a palliative scenario, in an attempt to maintain patient life quality [9]. Nevertheless, there are downsides and one of them is platinum agent-caused severe side effects, such as nephrotoxicity, ototoxicity, and peripheral neurotoxicity, limiting the dose that might be used for patient treatment [20]. Also, cancer survivors previously treated with platinum drugs disclose traceable levels of CDDP in urine and plasma after years of treatment, which is a major concern that may cause long-term side effects triggering a decline in quality of life, and ultimately increasing death risk [21-23]. The current Precision Medicine paradigm is no longer compliant with sustaining such side effects either in the short- and/or long-term and all efforts must be placed at improving risk stratification of patients with appropriate biomarkers, to spare patients from futile unnecessary treatments and their side effects.

1.1.2. Platinum-based agent structure and mechanism of action

CDDP or cis-diamminedichloroplatinum (II) is formed by one platinum atom bound to two chloride atoms and two amide groups (Figure 1., A) [24]. CDDP is known to cross the cell membrane by passive diffusion or through transmembrane transporters, e.g., the most widely studied copper transporters *CTR1* and *CTR2* [25, 26]. The cytosol favors the aquation process of CDDP due to low intracellular chloride concentration

entailing CDDP activation (Figure 1., B) [27]. Once inside the cell, CDDP is positively charged and strongly binds N7 reactive center of purine residues, causing DNA damage by forming intra-strand adducts (~90%) and inter-strand crosslinks (~10%) (Figure 1., B) or even binds proteins and forms DNA-protein crosslinks [28]. Following this scenario, it causes DNA damage by resulting in abnormal base pairing, excision of bases, and finally strand breakage, blocking cell division and resulting in apoptotic cell death [1, 29, 30]. It is important to highlight that only ~1% of CDDP that enters the cell binds DNA whereas the rest of the active drug may bind various cell proteins but, very extensively, enters mitochondria and induces apoptosis [31, 32]. The most critical CDDP side effect is nephrotoxicity which is related to renal proximal tubule cells having a high density of mitochondria [33, 34]. Due to the negative charge of mitochondria, positive CDDP metabolite accumulates within and causes damage, not only inducing apoptosis but also leading to severe side effects [33, 34].

CDDP analogs were and are being developed to have efficient platinum-based chemotherapy that may cause minimal side effects. One of the most used analog drugs is second-generation platinum agent carboplatin, chemically known as cis-diamino-(1,1-cyclobutandicarboxylate) platinum (II) (Figure 1., A). It differs from CDDP being more stable because of a six-membered ring which reduces its conversion to the aquated species rate. This results in lower toxicity and complicates platinum-DNA adduct formation in sensitive, for instance, kidney cells [35]. However, reduced feasibility of activation requires higher chemotherapy doses of carboplatin which eventually ends up in unwanted cytotoxicity and results in side effects like increased myelosuppression [36, 37]. Carboplatin is mostly used in combination with other chemotherapy agents for LC or OC treatment [36]. The mechanism of action of carboplatin is very similar to CDDP, and previous work has demonstrated that CDDP-resistant tumor cell lines are cross-resistant to carboplatin as well [36].

One more widely applied chemotherapy agent is third-generation platinum drug oxaliplatin, which has a 1,2-diaminocyclohexane carrier ligand (Figure 1., A) [38]. Unlike CDDP and carboplatin, oxaliplatin reacts rapidly in plasma, undergoing a process of transformation into reactive compounds, due to the displacement of the oxalate group [39]. The mechanism of oxaliplatin action is very similar to CDDP but oxaliplatin induces fewer though more lethal inter-strand crosslink lesions which end up in lower high-mobility-group (HMG) protein affinity to DNA [40]. It could explain why oxaliplatin has some activity in CDDP-resistant cells [41]. Single-agent oxaliplatin has low activity in

many tumors clinically, so it is often combined with other drugs such as 5-fluorouracil (5-FU) [42]. Currently, it is mostly used in the treatment of advanced CRC and CC [43, 44].

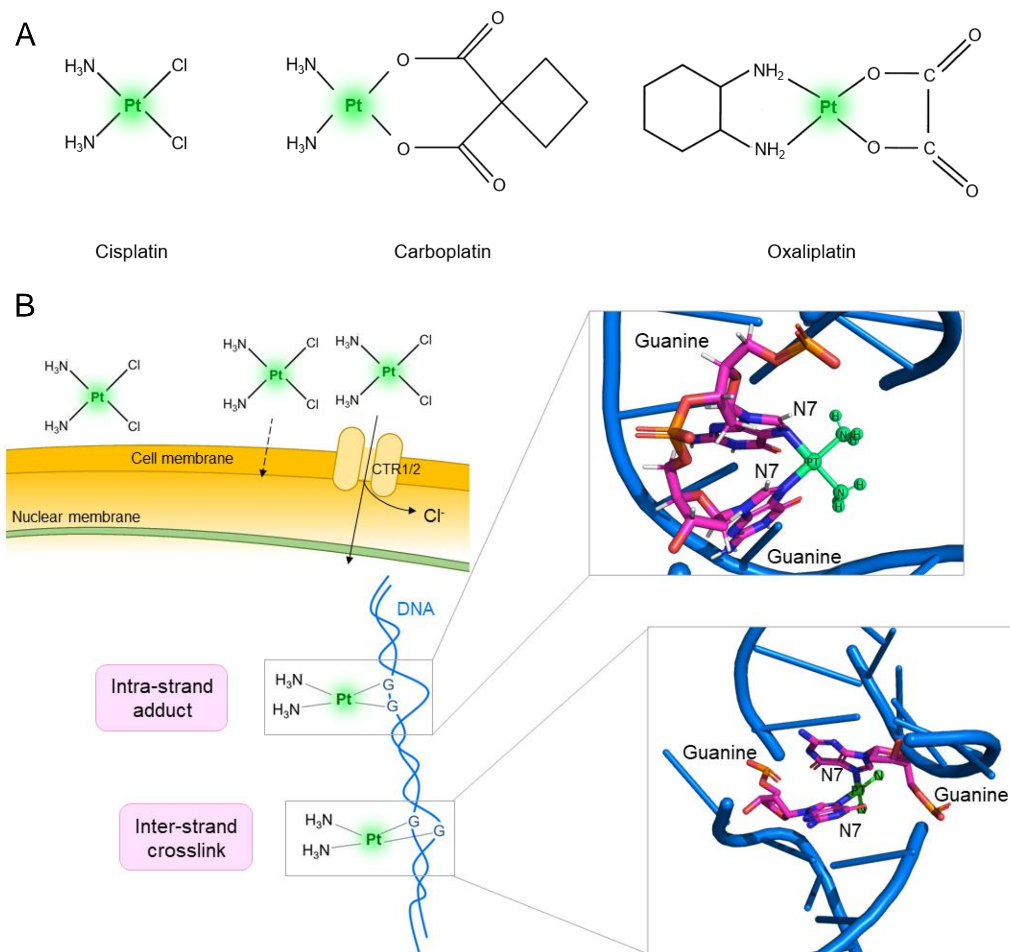


Figure 1. Molecular structure of CDDP, carboplatin, and oxaliplatin (A). Schematical representation of CDDP import into the cell through *CTR1* or *CTR2* channels (straight arrow) or by diffusion (dashed arrow) and formation of DNA intra-strand adducts or inter-strand crosslinks in the nucleus (B). Molecular representation of CDDP (balls and sticks) binding guanine N7 residues (licorice) on the same DNA strand or different DNA strands (B, right). Gumauskaitė, S., *unpublished*. Molecular representation was generated using PyMOL 2.5.2., the structures obtained from Protein Data Bank, NDBs 2NPW, and 1A2E.

1.2. Epigenetic cancer biomarkers

According to the National Cancer Institute (NCI) Dictionary of Cancer Terms, a cancer biomarker is a biological molecule, that is found in blood or other body fluids or tissues, which indicates an abnormal, cancer-related process or condition [45]. Biomarkers vary depending on their objective (risk assessment, diagnosis, prognosis, or prediction of response to therapy). Ideally, a biomarker should have perfect (100%) specificity (the ability of a test to correctly identify people without the disease) and

sensitivity (the ability of a test to correctly identify patients with a disease) [46, 47]. Specifically, predictive biomarkers come in handy for the identification of patients who would be more likely to respond to exposure to a particular medical intervention, e.g., a certain chemotherapy drug. The response here is defined as “symptomatic benefit, improved survival, or an adverse effect” [48]. A predictive value of the biomarker is relatively easy to determine by associating it with the clinical outcome though its’ establishment is followed by a set of challenges [49].

Overall, epigenetic biomarkers are influenced by genetic and environmental variance. Molecular structures like DNA methylation at the cytosine-phosphate-guanine (CpG) dinucleotides or amino acid residue modifications within histone proteins that determine the primary packaging of DNA can manifest themselves as epigenetic biomarkers [47]. CpG methylation might induce the occurrence of neoplasia by three major mechanisms, most often occurring simultaneously. The first one is hypomethylation which might enhance cancer cell division and prevent cell death by activating oncogenes. Second, focal hypermethylation at tumor suppressor gene (TSG) promoters silences them by reducing the access of transcription factors and results in increased tolerance of oncogene-related damage. The third one is direct mutagenesis of methylated cytosines by deamination or carcinogen exposure [50]. Even though CpG dinucleotide methylation in the malignant cells varies due to intratumor heterogeneity (ITH) in addition to existing intra-patient mutational heterogeneity, certain specific methylation patterns may serve as biomarkers [51, 52].

1.2.1. Application of epigenetic biomarkers in cancer

Recently, a lot of studies focus on the need to identify and select reliable biomarkers for aiding in the treatment of all cancer models. The patient sample (blood, urine, stool) contains circulating tumor DNA (ctDNA) which represents malignancy-related DNA methylation alterations. Patient sample collection is non-invasive and thus may be performed more frequently compared to tissue biopsy, which allows easier and earlier diagnosis, pre- and post-operative staging, and easy storage of patient samples [53]. In addition to this, it assists to create the treatment strategy, in an attempt to apply the most suitable neoadjuvant or adjuvant therapies [54].

In the context of the choice of a treatment method for a patient, the strategy of Personalized Medicine is becoming a priority. It is now well-known that there is no universal treatment for certain cancer model patients and some patients are resistant to specific kinds of treatment methods. The desired treatment strategy should target cancer cells in all the pathways that are crucial for their survival. Alterations in the epigenetic landscape are increasingly being considered one of the cancer hallmarks emphasizing their importance in neoplasia development (Figure 2.). Since epigenetic profile at least partly causes cancer resistance and relapse, epigenetic treatment comes into use, like the application of DNA demethylating agents [51, 55]. This is where epigenetic biomarkers become important, as they enable non-invasive patient sample collection, short analysis time, and good sensitivity to track cancer and evaluate whether a patient will benefit from, for example, CDDP-based chemotherapy.

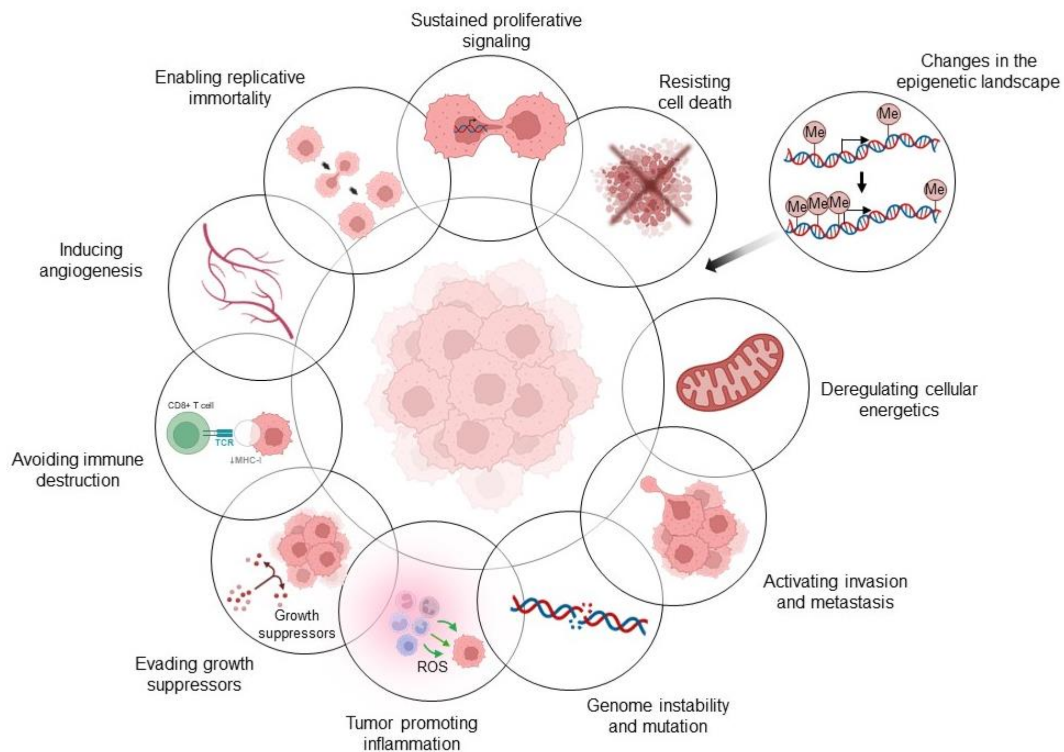


Figure 2. Hallmarks of cancer. Hanahan and Weinberg (2000) defined six essential alterations in malignant cells: sustained proliferative signaling, insensitivity to growth suppressors, evasion of apoptosis, replicative immortality, sustained angiogenesis, and tissue invasion/metastasis [56]. In 2011, the hallmarks were updated and new ones were confirmed [57]. Currently, more publications and a recent update of cancer hallmarks article also consider the importance of an emerging hallmark of Changes in the epigenetic landscape [58]. Abbreviations: MHC-I – major histocompatibility complex-I; ROS – reactive oxygen species; TCR – T cell receptor. Adapted from [58]. Gumauskaitė, S., *unpublished.* Image created with Biorender.com.

DNA methylation is advantageous compared to other epigenetic biomarkers not only because it may be detected with ctDNA from non-invasively collected patient

samples, but also because it represents the true tumor heterogeneity. While primary tumor and metastatic deposit tissue samples are highly heterogeneous and have several tumor cell clones that are missed by needle diagnostic biopsies sampling just a small part of the tumor area, circulating tumor cells or nucleic acids in the blood are representative of the tumor bulk, either primary or metastatic [51, 59]. Furthermore, either in tissue or liquid biopsy specimens, DNA is much more stable and resistant to degradation (considering sample conservation by formalin fixation, or thawing during sample processing) compared to RNA [47, 60, 61]. Finally, it is a convenient type of biomarker since the data obtained from DNA methylation detection may be compared to absolute reference points (fully methylated/unmethylated DNA) which allows performing quantitative assays [60].

Although such biomarkers seem auspicious due to their feasibility, they are still not widely used in practice owing to limited sensitivity compared to available standard of care tools. This is for the reason that often the straightforward detection of only a single biomarker (e.g., specific gene promoter hypermethylation) is not sufficient to obtain a reliable conclusion, which can be overcome by using gene panels. Additionally, for the purposes of validation, promoter methylation status must be first confirmed in several cohorts with distinct demographic features using multiple methods. Furthermore, also other environmental conditions may impact gene methylation acting as confounders in cancer biomarker studies [62, 63]. Additional issues are currently related to biomarker result reporting and interpretation, along the lines of normalization (appropriate normalizers, method of relative quantification), sample and DNA input conditions (which may be prohibitive in specific clinical scenarios), cost-related issues, etc.

1.2.2. The sequence of steps for reliable biomarker establishment

In order to set up a reliable biomarker that would be predictive of response to chemotherapy, one needs to identify relevant genes by compiling and testing training and validation cohorts and comparing DNA methylation status among certain cohorts of patients. Specifically, in this setting, the cohorts shall contain patients who responded (either completely or partially) to treatment and those who did not respond [47, 62, 64]. Additionally, it is crucial to adjust for demographic and clinicopathologic factors (age, gender, grade, stage, baseline characteristics of patients, etc.), due to the feasibility of

DNA methylation biomarkers to lose their predictive value after adjustment in multivariable models. Additionally, cancer cell line testing is a reliable method to be applied in the early steps of biomarker establishment. The main advantage of it is the ability to illustrate how chemotherapy-sensitive and -resistant cells react to treatment by evaluating whether DNA methylation profile changes over time and if the use of demethylating agent helps to sensitize resistant cells [65]. In the sequence of biomarker establishment steps, collection of patient tissue samples (for instance, biopsies, formalin-fixed paraffin-embedded (FFPE) tissue samples, frozen samples, etc.), DNA extraction (assuring the best possible quality), and bisulfite treatment (or variations, such as restriction with the use of methylation-sensitive endonucleases) followed by targeted MSP-based (methylation-sensitive PCR [polymerase chain reaction]) methods are required. Then, if it is observed that sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy reach high levels of performance, a biomarker is a great candidate for further testing in body fluids to determine whether it will finally constitute a reliable biomarker for clinical use [60].

In summary, as reviewed in Henry and Hayes, 2012, an established biomarker should distinguish itself with analytic validity (ensure proper sample handling, reproducibility, parameters like sensitivity and specificity), clinical validity (biomarker characteristic of the population divided into two distinct groups) and clinical utility (effectiveness and benefit-to-harm ratio) [66].

1.3. Epigenetics and the role of DNA methylation

It is well-known that cancer may be caused by genetic changes in cells but additionally, epigenetic alterations must be taken into account due to high importance. The radical difference between genetic and epigenetic changes is that genetic lesions are irreversible whereas epigenetic lesions are potentially reversible, associated with gain or loss of DNA methylation or other modifications of chromatin, allowing therapeutic intervention [67, 68].

Epigenetic mutations, also called epimutations, are heritable, they might be constitutional being derived from germline and expected to be in all of the tissues of an individual or they might be somatic, appearing in a somatic tissue [69]. Epigenetic changes are crucial to understanding because they impact all human cancer models and

collaborate with genetic lesions to generate a malignant phenotype. Epigenetic marks are various and disclose as abnormal patterns of DNA methylation, disrupted patterns of histone posttranslational modifications (PTMs), small non-coding RNAs, and alterations in chromatin composition and/or organization [50]. Histone modification and DNA methylation regulate gene expression at the transcription level and small noncoding RNAs regulate at the translation level, indicating the extensiveness of epigenetic effects in several magnitudes of regulation [70].

1.3.1. DNA methylation effects on transcription and protein levels

DNA methylation is a widely studied epigenetic mechanism of gene silencing. Shortly after the discovery of the methylated cytosine base in DNA resulting in 5-methylcytosine (5mC), its role in oncogenic phenotype started to be investigated [71, 72]. The best-studied and most common is CpG dinucleotide methylation. Non-CpG methylation also occurs though at lower levels and is not so highly cancer-related [70].

Mammals have three DNA methyltransferase enzymes performing slightly different functions: *DNMT1* (responsible for maintenance of methylation), *DNMT3A*, *DNMT3B* (responsible for *de novo* methylation) [73]. Methylation is mostly observed in CpG islands which often contain transcriptional start sites [74]. This type of promoter-related methylation ensures stable silencing of gene expression, reducing the accessibility of DNA and diminishing transcription factor binding in 5' regions of genes related to neoplasia like TSGs. However, methylation in the gene body may also alter gene expression and induce cancer progression but this phenomenon is yet to be more investigated [75].

Hypermethylation negatively impacts transcription and thus reduces levels of proteins responsible for processes like DNA damage repair or apoptosis, creating an opportunity for the exact purpose of neoplasia to have a fundamental replication advantage against normal tissue cells [76].

1.3.2. Epigenetic resistance to chemotherapy with platinum-based agents

Some of the main efforts of research in platinum-based chemotherapy have been the development of new CDDP analogs with fewer side effects, especially the ones that might evade the resistance to the treatment. Resistance to platinum treatment may be divided into two different types: intrinsic or acquired. Some patients represent intrinsic resistance to the drugs and do not respond to the treatment initially, being exposed to severe side effects. Many patients that initially are sensitive to the treatment develop resistance to it during their treatment course, which causes relapse and reduces its overall clinical efficacy [27]. This acquired resistance is known to be of genetic and epigenetic origin. For example, in the scope of OC, 10-15% of patients are intrinsically resistant to platinum-based agents and even 80% of patients might represent acquired resistance over the treatment course [77].

Changes in the epigenetic landscape appear to be nonrandom and are associated with the acquisition of chemoresistance to platinum in various types of cancers [78, 79]. Thus, according to several previous studies, platinum-based chemotherapy may induce changes in DNA methylation [80-84]. This epigenetic mechanism plays a big part in the resistance by affecting the transcription and translation of genes that are involved in pathways that reduce platinum influx into the cell, increase export (e.g., *ABCB1* [85]), heighten platinum detoxification (e.g., *MT1E* [86]), enhance DNA damage repair routes (e.g., *BRCA1* [87], *ERCC1* [88], *MLH1* [89]) and higher tolerance of DNA damage, inactivate apoptosis pathways (e.g., *Casp8AP2* [90], *p73* [91]), alter pleiotropic processes and pathways (e.g., *CFLAR* in NFκB signaling [92, 93]), or change the tumor microenvironment (e.g., *COL1A1* in extracellular matrix remodeling [94]) [95] (Figure 3.).

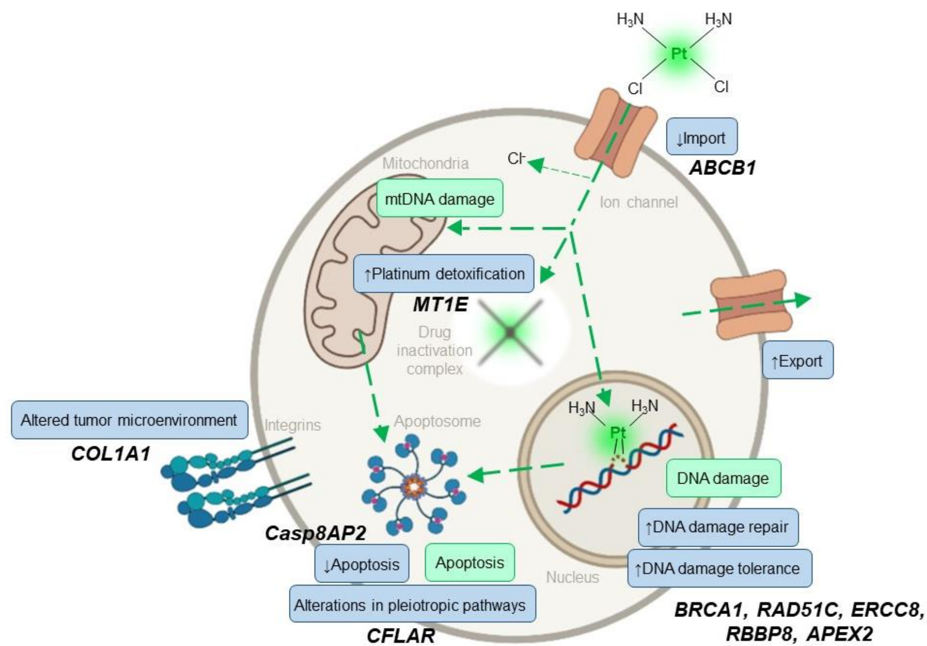


Figure 3. Platinum-based agent anti-neoplastic mechanisms and resistance pathways. Green arrows indicate the most common pathways of platinum drugs (specifically, CDDP) inside a cell. It is represented how CDDP enters the cell through an ion channel (e.g., *ABCB1*) and Cl^- ions dissociate, leading to CDDP activation so it may further follow the pathways highlighted. Green rectangles specify the main processes of CDDP damage exertion inside a cell. Blue rectangles indicate the main CDDP resistance pathways together with example genes (in italic) that participate in the specified pathways. Abbreviations: ↑ - increased; ↓ - reduced. Adapted from [95]. Increased DNA damage repair and tolerance genes are the ones investigated in this thesis dissertation. Gumauskaitė, S., *unpublished.* Image created with Biorender.com.

Table 1. lists accessible studies with patient samples of promising DNA methylation markers which are predictive of resistance to platinum-based chemotherapy. Hence, the cancer models included in the table are only the ones investigated in this thesis dissertation (BC, GC, CRC, LC, TGCT, EC). The studies listed represent the extensiveness of research on DNA methylation biomarkers for the prediction of resistance, though the sensitivity and specificity have to be improved for these genes to be successfully applied in clinics and their use still needs to prove reliable in human plasma samples.

Table 1. Promising DNA methylation markers predictive of resistance to platinum-based chemotherapy in BC, EC, GC, CRC, LC, TGCT (studies with patient tumor samples). Adapted from [96].

Gene(s)	Platinum compound	Tumor model	Sample Type	Sample Grouping and Size	Patients' Gender and Mean Age	Methylation Detection Method	Key Findings	Ref.
TLX3	CDDP	BC	Tumor tissue samples	110 patient samples	n.m.	RLGS; COBRA; bisulfite sequencing	*TLX3* was hypermethylated in tumors resistant to CDDP. Results in patient samples and cell lines were congruently indicating a role for *TLX3* as a marker of CDDP response.	[97]
HOXA9	CDDP	BC (MIBC)	Tumor tissue samples from vesical transurethral resections	18 patient samples	15♂ and 3♀; mean age 69 y.o. at the time of cystectomy (median 71, range 60 to 77 y.o.)	EpiTYPER™ assay	*HOXA9* promoter methylation status was associated with response to CDDP-based chemotherapy in MIBC. *HOXA9* promoter methylation might be used to predict sensitivity or resistance to CDDP-based chemotherapy in BC patients.	[98]
GULP1	CDDP	BC (UC)	Tumor tissue and urine samples	46 healthy control urine samples; 58 of UC patients; 20 primary tumors and matched normal samples; 76 primary tumors	n.m.	MSP	qMSP in tumor samples showed a significantly higher frequency of *GULP1* promoter methylation in tumors than in matched normal tissues. The results were confirmed in urine samples and TCGA-BC dataset. *GULP1* may be a biomarker of resistance to CDDP.	[99]
p73	CDDP	BC (MIBC)	Tumor tissue samples	14 patient samples (8 low and 6 high methylation)	n.m.	Infinium® HumanMethylation450K BeadChip; pyrosequencing	*p73* promoter methylation was significantly related to worse OS (high-methylation: 13.5 months vs low methylation: 30 months). *p73* promoter hypermethylation might be a predictive biomarker for CDDP response in BC patients.	[91]
SLFN11	CDDP	CRC	Tumor tissue samples	133 patient samples (128 primary cases and 5 noncancerous colorectal mucosae)	84♂ and 44♀ (30<50 y.o. and 98≥50 y.o.)	MSP; bisulfite sequencing	*SLFN11* was found methylated in 55.47% of CRC samples, regulating gene expression. *SLFN11* methylation is significantly associated with age, poor 5-year OS, and RFS.	[100]
TFAP2E	Oxaliplatin	CRC	Tumor tissue samples	74 patient samples (metastatic CRC)	n.m.	MethylLight	The cohort treated with oxaliplatin disclosed a negative association between methylation and treatment response – higher response rates among patients with hypomethylated *TFAP2E* (3/20 patients with hypermethylated *TFAP2E*	[101]

							responded to treatment, whereas 33/54 patients with hypomethylated *TFAP2E* responded).	
FGF5	CDDP	EC (ESCC)	Tumor tissue samples	117 patient tumor samples of responders and non-responders (41 patients in screening, 44 in validation, 42 in re-validation set)	Screening set: 34♂, 7♀ (mean age 64,6 y.o.); validation set: 28♂, 6♀ (mean age 66,8 y.o.); re-validation set: 30♂, 9♀ (mean age 65,9 y.o.)	Infinium® HumanMethylation450K BeadChip; bisulfite sequencing	*FGF5* methylation may be a biomarker predictive of sensitivity to CDDP dCRT. Methylome screening identified the specificity of *FGF5* expression and associated promoter methylation levels with the response (45% sensitivity and 90% specificity in the combined validation and re-validation sets, n=76).	[102]
PAX5	CDDP	EC (ESCC)	Tumor tissue surgical samples	156 ESCC patient samples (78 tumor and 78 normal adjacent)	62♂ and 16♀, 37≥65 y.o. and 41<65 y.o.	qMSP	*PAX5* methylation was frequent and highly tumor-specific in ESCC. Methylation was significantly associated with low protein expression in tumors. *PAX5* silencing correlated with increased cancer cell proliferation, CDDP resistance and might associate with poor RFS.	[103]
BMP4	CDDP	SC	Tumor tissue samples	197 patient samples	n.m.	Bisulfite sequencing; MSP	A significant correlation between *BMP4* methylation status and mRNA expression was found across tumors investigated. *BMP-4* expressing tumors were associated with poor GC prognosis and possible resistance to CDDP.	[104]
MLH1	Oxaliplatin	SC	FFPE tumor tissue samples	53 oxaliplatin-treated patient samples	72♂ and 30♀, median age 53 y.o.	Nested MSP	*MLH1* promoters were methylated in 30.2% cases of oxaliplatin-treated patients. OS was higher in the unmethylated vs. methylated group (p=0.046). Patients with methylated *MLH1* were resistant to oxaliplatin. The methylation may be an oxaliplatin-resistance marker in GC.	[105]
SLFN11	CDDP	SC	Tumor tissue samples	209 patient samples (201 GC samples and 8 normal gastric mucosa samples)	157♂ and 44♀ (39 patients <50 y.o. and 162 patients ≥50 y.o.)	MSP; bisulfite sequencing	*SLFN11* was methylated in 29.9% of human SC samples and *SLFN11* expression was regulated by promoter methylation. *SLFN11* methylation was significantly associated with tumor size.	[106]
APC, RASSF1A, HIC1, BRCA1, MGMT, RARB, FHIT, FANCF, ECAD	CDDP	Male GCT	Tumor tissue samples	70 patient samples (31 CDDP-sensitive and 39 resistant)	n.m.	MSP	One or more genes were methylated in 59% of tested tumors. The top hypermethylated genes were *RASSF1A* (35.7%), *HIC1* (31.9%), *BRCA1* (26.1%), *APC* (24.3%). *RASSF1A* and *HIC1* inactivation by promoter hypermethylation may be resistance markers.	[107]

GDA, S100P, WISP2, LOXL1, TIMP4, ICAM1, HSP8, GAS1	CDDP	NSCLC	Primary tumor samples	40 patient samples (20 CDDP-resistant and 20 -sensitive)	n.m.	Infinium® HumanMethylation450K BeadChip platform; qMSP	The genes were found to disclose higher methylation levels in CDDP-resistant samples compared to sensitive tumors.	[108]
IGFBP-3	CDDP	NSCLC	Paraffin-embedded surgical specimens	36 patient samples (19 CDDP-resistant and 17 sensitive); 10 control biopsies	34♂ and 2♀; mean age 65.8 y.o.	Bisulfite sequencing; MSP	Most CpG dinucleotides were methylated only in resistant primary tumors, indicating a significant association between *IGFBP-3* methylation and CDDP chemosensitivity.	[109]
IGFBP-3	CDDP	NSCLC	Paraffin-embedded surgical specimens	25 patient samples	23♂ and 2♀; mean age 63.7 y.o.	MSP	*IGFBP-3* promoter methylation and *IGFIR/AKT* phosphorylation occurred only in CDDP-resistant patients. *IGFBP-3* deficiency due to methylation may mediate the resistance to CDDP through activation of *IGFIR/AKT* pathway.	[110]
LRP12	Carboplatin	NSCLC	FFPE primary tumor samples, and frozen tumor tissue samples	PDX models derived from 22 patients; validation in an independent cohort of 35 patient FFPE samples	n.m.	Me-DIP Seq; targeted bisulfite sequencing; MSP	*LRP12* hypermethylation correlated with increased resistance to carboplatin. *LRP12* methylation was significantly higher in patients with relapse (13.9% vs 7.4%). A threshold of 8.3% was determined for classifying tumors into treatment responders and non-responders (80% sensitivity, 84% specificity).	[111]
RIP3	CDDP	NSCLC	Frozen tumor tissue samples	16 NSCLC patients (both normal and tumor tissues)	n.m.	COBRA; Infinium® HumanMethylation450K BeadChip	The quantitative methylation data for probes located within the *RIP3* promoter CpG island revealed significantly higher methylation in 25% of tumors. *RIP3* promoter methylation correlated with suppressed protein expression and increased resistance to CDDP.	[112]
SLFN11	CDDP, carboplatin	NSCLC	Tumor tissue samples	22 patient samples	10♂, 12♀ (2<50 y.o., 20>50 y.o.)	Infinium® HumanMethylation450K BeadChip; bisulfite sequencing	*SLFN11* hypermethylation was associated with shorter OS and PFS. Clinical results paralleled those of cancer cell lines.	[113]

Abbreviations: BC – bladder cancer; COBRA – combined bisulfite restriction analysis; dCRT – definitive chemoradiotherapy; EC – esophageal carcinoma; ESCC – esophageal squamous cell carcinoma; FFPE – formalin-fixed paraffin-embedded; SC – stomach cancer; Me-DIP-seq – Methylated DNA immunoprecipitation sequencing; MIBC – muscle-invasive bladder cancer; n.m. – not mentioned; MSP – methylation-specific PCR; NSCLC – non-squamous cell lung carcinoma; OS – overall survival; PDX – patient-derived xenograft; PFS – progression-free survival; qMSP – quantitative methylation-specific PCR; RFS – relapse-free survival; RLGS – restriction landmark genomic scanning; TCGA – The Cancer Genome Atlas; UC – urothelial carcinoma; y.o. – years old.

1.3.3. Role of specific DNA repair genes in platinum-based agent resistance

Alkylating agents exert their cytotoxic effects principally through nuclear DNA damage so DNA repair machinery is highly involved in the response of platinum-based chemotherapy [28] and this dissertation is specifically focused on several DNA repair genes. In total there are at least five major DNA repair pathways [114] and four of them, excluding base excision repair (BER), are related to fixing exogenous DNA damage caused by alkylating agents – nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end-joining (NHEJ) [24]. Promoter methylation and thus inactivation of genes implicated in DNA repair might underly an enhanced response to platinum compounds. Eventually, hypermethylation of certain DNA repair genes might become a biomarker, indicating patient resistance to platinum-based agent chemotherapy treatment. An example illustrating the importance of the absence of DNA repair genes and sensitivity to alkylating agents is testicular cancer – data suggests that the high sensitivity of this cancer model patients to CDDP is due to DNA repair deficiency in testis cells [24]. On the other hand, hypomethylation of DNA repair genes can also be related to resistance to alkylating agents due to dynamic crosstalk between several genes [115].

Double-strand break (DSB) repair (HR, NHEJ) mechanisms are especially involved in the process of fixing platinum agent-caused DNA damage and their deficiency has been proven to increase tumor immunogenicity [116, 117]. DSBs may appear due to alkylating agents or originate in the inter-strand crosslink repair process [24]. Even though DSBs are less common than single-strand adducts, their effect on the cell is more severe [118]. There is evidence that combinatorial chemotherapy of DSB repair inhibitors with DNA-damaging agents (e.g., alkylating agents) presents high effectiveness in the treatment [117]. This highlights targetable tumor vulnerability which may be used for the development of treatment strategies.

On the other hand, single-strand repair pathways (MMR, NER) also play an important role in fixing platinum drug-caused damage. Reduced promoter methylation of NER genes like *ERCC8* could explain platinum agent resistance, meaning the malignant cell might more efficiently repair the damage [119]. Additionally, altered methylation of MMR genes like *MLH1* might indicate resistance. Since *MLH1* is a TSG, its hypermethylation and consequently silencing would be crucial for neoplastic cell survival [120].

A less explored DNA repair mechanism that might be involved in platinum-based agent resistance mechanism is the BER pathway. BER is an endogenous DNA damage repair process but the proteins responsible for this pathway have been implicated in other repair mechanisms. To illustrate, *APEX2* might be related to the regulation of HR in multiple myeloma (MM) by regulating *RAD51* expression [121]. Additionally, *APEX2* might be relevant for the viability of *BRCA1*-deficient cells by reversing blocked DNA 3' ends and reversing lesions that preclude DNA synthesis [122].

Finally, apart from the cancer cells being able to repair drug-induced DNA damage, they may also tolerate it. This might be due to a specific class of DNA polymerases that are responsible for translesion synthesis [123]. Increased tolerance to DNA adducts might also be observed in cases when there is a deficiency in MMR genes [124].

1.4. Epigenetic therapy

Since epigenetic changes in DNA are reversible, they provide promising targets for a specific treatment, so-called epigenetic therapy. The epigenetic landscape of normal tissue and tumor cells is different allowing to target malignant cells. The desired use of epigenetic biomarkers is their detection, evaluation, and use for compiling personalized treatment strategies with the least side effects (Figure 4.). In the case of DNA hyper/hypomethylation, epigenetic therapy might be used to alter the epigenetic landscape and avoid patient overtreatment, unnecessary side effects, and drug resistance development. Currently, there is a variety of epigenetic drugs that target epimutations on several levels. For example, certain drugs may target bromodomains which are structural motifs common for chromatin-modifying proteins, others function by inhibiting histone acetylases or deacetylases, protein methyltransferases, histone methylation, or function as methylation inhibiting drugs [125].

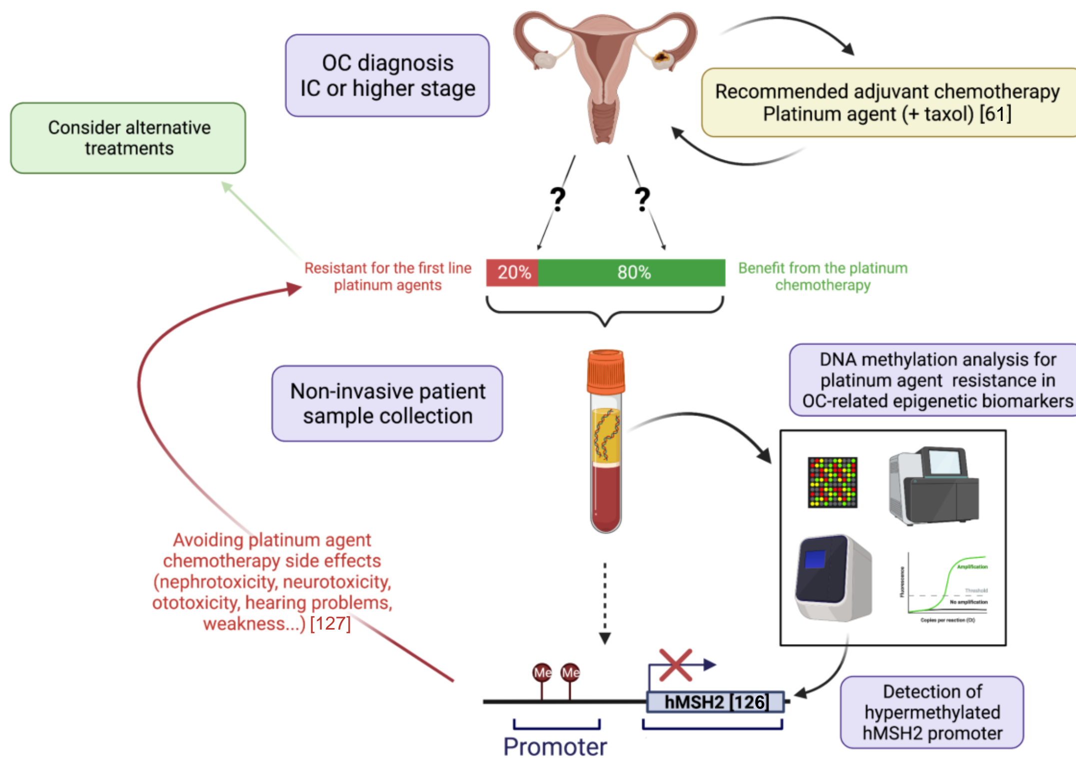


Figure 4. Schematic representation of pipeline for validation of DNA methylation-based biomarker to predict resistance to platinum-based chemotherapy in ovarian cancer (OC) patients. After a clinical diagnosis of OC (top of the picture), if the disease was staged as IC or higher, the recommended treatment is adjuvant chemotherapy with a platinum agent (CDDP, carboplatin, or oxaliplatin), eventually in combination with Taxol. However, there is a 20% probability that the patient will be resistant to platinum agents which complicates the choice of treatment [61]. To select the best treatment method, biomarker validation could be performed. This follows with non-invasive patient sample collection (for instance, blood plasma) which can be used for circulating tumor DNA methylation analysis, focusing on platinum agent resistance. In this case, gene promoter hypermethylation indicating platinum resistance in OC was detected (e.g., *hMSH2*) [126], indicating that the patient will likely endure platinum resistance. Thus, not only the side effects of ineffective treatment [127] may be avoided, but also alternative treatments, eventually including epidrugs, should be considered. Adapted from [96].

1.4.1. Methods for DNA methylation detection

There is a large number of methods to detect DNA methylation, either target-based (e.g., MSP, bisulfite sequencing, pyrosequencing methylation-specific restriction endonucleases, etc.) or genome-wide-based (e.g., 450K or 850K array) and a vast amount of data are available publicly, enabling comparisons [62]. Finally, improvements in technology are under development to facilitate the detection of DNA methylation biomarkers in an absolute way without the need for pre-amplification reactions (e.g., droplet digital PCR [ddPCR]) [128, 129]. In the end, the desired aim is to choose a sensitive and direct method to determine the methylation state of biomarkers in patient samples.

1.4.2. Epigenetic therapy by methylation inhibition

Focusing on epigenetic therapy by methylation inhibition, there are several kinds of epigenetic drugs that interfere with the methylation process. Most common are nucleoside-like compounds, also called demethylating agents due to their action mechanism, e.g., 5-azacytidine (5-aza), and decitabine which are approved by FDA. 5-aza is a cytidine analog that contains nitrogen atom instead of carbon in the 5th position. Inside the cell, during replication, it is as usual incorporated into DNA and recognized by *DNMT1* but due to the nitrogen group, it forms an irreversible DNMT1-aza complex, leading to the degradation of the enzyme and reduction of overall methylation in rapidly dividing malignant cells [130]. Decitabine is another cytidine analog that, in addition to having nitrogen instead of carbon in the 5th carbon of the pyrimidine ring, does not have one of the hydroxyl groups of the ribose, compared to cytidine. The difference between the two epigenetic drugs is potency because 5-aza is predominantly incorporated into RNA whereas decitabine is 10 times more potent, is only incorporated into DNA [131]. MG98 is an example of an antisense oligonucleotide epigenetic drug, which is still under clinical trials. It binds the 3' untranslated region of *DNMT1*, preventing its transcription [132]. RG108 is a small *DNMT1* non-nucleoside inhibitor molecule that is under investigation for future use, as an epigenetic drug with low cytotoxicity [125, 133].

Although there are a lot of promising epigenetic drugs under clinical trials and investigation, epigenetic therapy is a field that represents a lot of challenges. Epigenetic plasticity enables neoplastic cells to develop tumor heterogeneity, increasing its malignancy. On the other hand, it complicates the epigenetic therapy process due to tumor cells being able to adapt to the stress or overcome it and avoid the destructing effect of chemotherapy drugs [134]. However, epigenetic drugs have been more widely applied together with chemotherapeutics to produce epigenetic priming of the malignant cells to make them more chemotherapy sensitive [135], thus reducing side effects in addition to avoiding the development of resistance [136-138] and already in the near future they might be involved in treatment strategies, constructed based on the biomarkers.

2. AIMS

Platinum-based agents are broadly used for chemotherapy treatment in several types of cancer. Certain challenges in the use of platinum agents, specifically drug resistance, require investigation to better understand the mechanism of action of these compounds. DNA methylation-based biomarkers have been suggested to be able in assisting the selection of patients aiding to foresee their platinum therapy response. These kinds of biomarkers show increasing importance, as they enable non-invasive patient sample collection, short analysis time, and good sensitivity. Hence, improved methodologies for their detection and quantification are attractive features that can bring these biomarkers into clinical practice, fostering Precision Medicine.

Thus, the main objective of this dissertation is to test a group of 5 DNA repair-related genes identified by a preliminary *in silico* analysis at TCGA in a retrospective series of platinum-based agent-treated patient tissue samples who showed differential clinical responses (responders/partial responders *versus* poor responders/non-responders) in order to investigate most promising DNA methylation predictive biomarkers.

Namely, the specific tasks of the project were:

1. Design specific qMSP primers and probes to assess methylation levels of previously *in silico* determined most promising six DNA repair gene (*BRCA1*, *RAD51C*, *RBBP8*, *ERCC8*, *APEX2*) promoters;
2. Select EC, TGCT, LC, CRC, SC, and BC patient FFPE tissue samples corresponding to both tumor tissue before treatment with platinum-based agents and after the treatment;
3. Extract DNA from FFPE tissue samples, perform sodium bisulfite treatment and multiplex qMSP;
4. Determine the predictive performance of gene methylation levels for each cancer type and assess their putative value as predictive biomarkers.

3. MATERIALS AND METHODS

3.1. Study group

A study cohort of total 153 Portuguese Oncology Institute of Porto (IPO Porto) patients who received neoadjuvant chemotherapy (in case of TGCT – adjuvant chemotherapy) containing platinum-drug (CDDP, carboplatin, or oxaliplatin) between 2005 and 2020 consists of 10 BC patients, 26 EC, 41 SC, 30 CRC, 27 LC, and 19 TGCT patients (patient data collection for the study explained in Appendix I). All samples were derived from routinely archived material (FFPE tissues of surgical specimens) which were used after anonymization. This study was approved by the institutional Ethics committee (CES-IPO-241/021). All the samples were obtained from the IPO-Porto hospital where the cohort of patients had undergone resections of primary tumors. Pre-chemo tissue samples were biopsies from primary tumors, post-chemo samples were resected tumor tissues of poor/partial responders, and complete samples were blocks from surgical margins of patients after treatment without relapse. Relevant clinical data were retrieved from clinical files.

Histological classification and tumor staging were re-coded in accordance with 2022 The WHO Classification of Urinary and Male Genital Tumours [139], 2019 The WHO Classification of Digestive System Tumours [140], 2021 The WHO Classification of Thoracic Tumours [141], and the 8th edition of the American Joint Committee of Cancer (AJCC) staging manual [142].

EC, SC, CRC, and LC patients were categorized into poor, partial, and complete responders following the tumor regression grading (TRG) system categories defined in pathology reports (AJCC/CAP recommendations). BC and TGCT patients were divided into relapsers and non-relapsers following their state on the most recent clinical follow-up.

3.2. Histopathologic evaluations

Haematoxylin and eosin (H&E) stained tissue sections of all the samples were examined under a light microscope by a pathologist to ensure the type of tissue. Additionally, during the examination delimitation of the areas was performed for further DNA purification from the most representative regions of FFPE slides.

3.3. Genomic DNA extraction

DNA was extracted from FFPE tissue samples, using the FFPE DNA Purification Kit (Norgen Biotek Corp., Canada), according to the manufacturer's instructions (Figure 5.).

For each patient, one paraffin-embedded tissue block was considered and 5 or 7 serials (depending on tumor sample availability) 8 µm thick unstained slides and one H&E-stained slide were prepared by a technician. As described before, the H&E-stained slides were examined and the area of interest was delimitated. Subsequently, the tumor areas were delimited by comparison with the correspondent H&E-stained slides and macro-dissected from the tissue unstained slides.

Briefly, the macro-dissected FFPE tissue sections were transferred into a Nuclease-free microcentrifuge tube, 1 mL of xylene (VWR™ BDH Chemicals, Avantor®, Radnor, PA, USA) was added and the sample was vortexed, following incubation in 50°C for 5 minutes in thermoblock Thermal Shaker Touch (VWR™, Radnor, PA, USA). After incubation, the sample was centrifuged in Fresco™ 17 Microcentrifuge (Thermo Scientific™, Waltham, MA, USA) at 14,000 × g for 2 minutes (all centrifugations were carried out at room temperature (RT), 20°C). Xylene was carefully removed without dislodging the pellet and consequently, the pellet was resuspended in 96 – 100% ethanol (VWR™ BDH Chemicals, Avantor®, Radnor, PA, USA) and mixed by vortexing following centrifugation at 14,000 × g for 2 minutes. The washing with 96 – 100% ethanol was performed two times and finally, the pellet was air dried in room temperature (RT) until completely dry (up to 3 hours).

The lysate was prepared by adding 300 µL of Digestion Buffer A, 10 µL of reconstituted Proteinase K, and 1 µL of RNase were added, consequently mixing by vortexing. The suspension was incubated at 55°C for 1 hour agitating at 900 rpm, followed by 90°C for 1 hour continuing the agitation in thermoblock Thermal Shaker Touch (VWR™, Radnor, PA, USA). Afterwards, 300 µL of Buffer RL were added, the mixture was vortexed, and 250 µL of 96 – 100% ethanol was added also followed by vortexing.

Afterwards, DNA was bound to the column by applying 600 µL of the clarified lysate from the previous step onto the column assembled with a collection tube and centrifugated at 14,000 × g for 1 minute. After centrifugation, the flowthrough was discarded and the rest of the lysate was added to the column, following centrifugation at 14,000 × g for 1 minute.

The washing of the column was performed by adding 400 µL of Wash solution A to the column and centrifuging at 14,000 × g for 1 minute. The flowthrough was discarded and the

column was washed with Wash solution A for two times more. After the third wash, the column was spun empty at 14,000 × g for 2 minutes and the column eventually was placed into a fresh 1.7 mL Elution tube.

For the elution step, 10 µL of Elution Buffer B were added to the column, and the assembly was incubated in RT for 1 minute and centrifugated at 14,000 × g for 1 minute. Afterwards, another 10 µL of Elution Buffer B was added to the column, followed by the same incubation and centrifugation, obtaining the final volume of 20 µL of purified DNA solution. Finally, DNA was quantified and its purity was assessed using NanoDrop ND-1000 ® (NanoDrop Technologies, DE, USA) spectrophotometer. All the samples were stored at -20°C until further use.

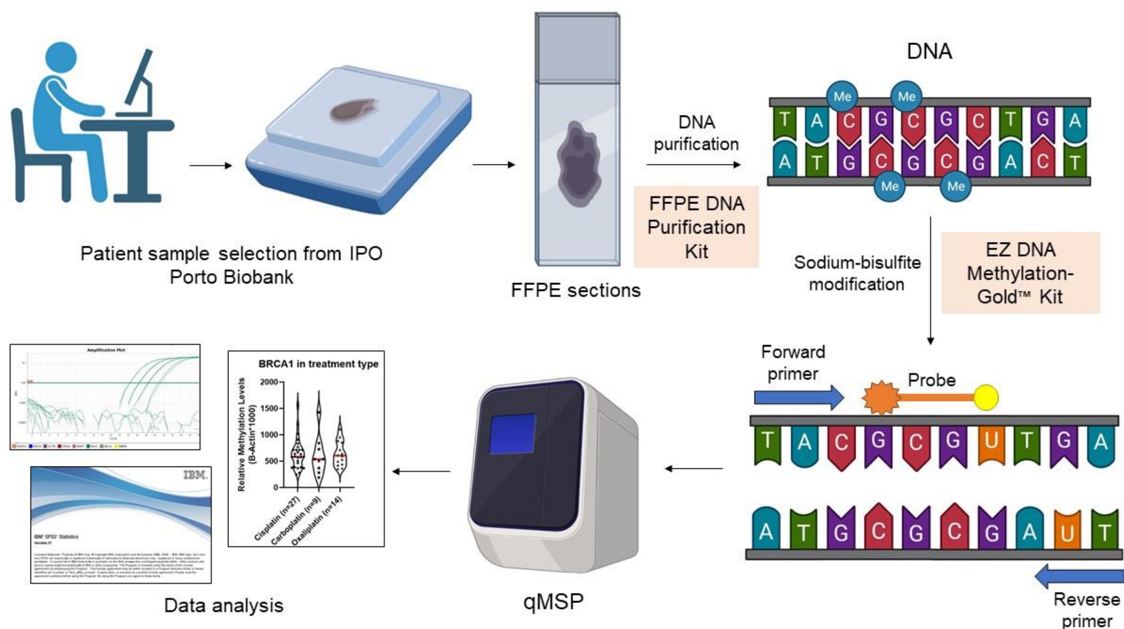


Figure 5. Overview of the techniques performed. Patient tumor samples were selected from IPO Porto Biobank database and FFPE sections of patient tumors were requested from technicians. Then, the slides were inspected by a pathologist, and DNA from the most representative tumor regions was purified and quantified. Consequently, sodium-bisulfite modification was performed to convert unmethylated cytosines into uracils, while maintaining methylated cytosines unchanged. Lastly, promoters' methylation levels were assessed by multiplex qMSP, using previously created and optimised primers and TaqMan probes and finally the data analysis was performed with SPSS software and the graphs were designed with Graphpad software. Gumauskaitė, S., *unpublished.* Created with BioRender.com.

3.4. Sodium-bisulfite modification

Sodium-bisulfite modification (Figure 5.) is a pivotal gold-standard technique for DNA methylation analyses. This method includes the treatment of DNA with bisulfite, which

converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged, based on three consecutive chemical reactions (sulphonation, deamination, and desulphonation) [143]. Therefore, after conversion, the DNA methylation profile can be determined by MSP and sequencing, through the design of primers and TaqMan probes specific for the modified DNA sequence.

All FFPE purified DNA samples were sodium-bisulfite modified using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's recommendations. Firstly, 130 µL of CT conversion reagent solution was added to 20 µL of the previously extracted DNA of each sample. Then, samples were incubated at 98ºC for 10 minutes for DNA denaturation, followed by 64ºC for 180 minutes for bisulfite conversion reaction in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). Next, each sample and 600 µL of M-Binding Buffer were added to Zymo-Spin™ IC column and incubated for 10 minutes. The columns were centrifugated at 10,000 rpm for 30 seconds. Following the addition of 100 µL of M-Wash Buffer and centrifugation, 200 µL of M-Desulphonation Buffer were added to the column, followed by 20 minutes incubation and centrifugation at 10,000 rpm for 30 seconds. After, the column was washed twice with 200 µL of M-Wash Buffer and centrifugated at 10,000 rpm for 30 seconds. Finally, the columns were transferred to 1.5 mL safe-lock tubes and 10 µL of sterile distilled water were added to elute the bisulfite-converted DNA. Following a 5 minutes incubation, the columns were centrifugated at 12,000 rpm for 30 seconds. This process was repeated twice. All steps were performed at RT. 1 µg of positive and negative control from Human methylated & Non-methylated DNA Set (Zymo Research, Irvine, CA, USA) was also modified using the previously mentioned protocol and eluted in 30 µL of sterile distilled water (final volume 60 µL). The bisulfite-converted DNA was stored at -80ºC until further use.

3.5. *In silico* analyses: selection of DNA repair genes and most relevant CpG sites

Initial *in silico* analysis for the selection of DNA repair genes and most relevant CpG sites in their promoters for the study was kindly performed by Vera Constâncio, João Lobo, and Pedro Leite-Silva using TCGA dataset.

3.6. Design of primers and probes for detection of methylated CpG sites

The sequences of each of the target genes were downloaded from the UCSC Genome Browser [144]. The search parameters for obtaining the promoter sequence of each gene: "Promoter/Upstream by 1500 bases", including "5' UTR Exons", "CDS Exons", "3' UTR Exons". In order to distinguish the promoter for further analysis, the sequence up to the first ATG in the first exon was chosen, using the codon as an identifier of the start of the reading frame. Further on, the CpG sites previously determined *in silico* were located in the obtained promoter sequence by using SMART App [145] to find their coordinates and Genome Browser to localize them in the promoter nucleotide sequence.

Bisulfite treatment for methylated DNA promoter sequence was simulated using Methyl Primer Express™ v1.0 (Applied Biosystems™, Waltham, USA) software. The obtained modified sequence was used for the design of qMSP primers and TaqMan probes. Primers were designed for the final product to be a promoter region of 80-150 base pairs and meet the main objective for primers and probe in each product to overlap with ~6 CpG sites of interest (selected *in silico*) and ~8 cytosines that do not belong to CpGs. Each of the probes and pairs of primers were constructed to have CG content of 30-80%, probes having ~10 °C higher melting temperature (T_m) than the primers (58-60 °C), and the length of primers being ~20 and probes 13-30 nucleotides. The T_m of designed primers and probes were evaluated with Primer Express™ 3.0 (Applied Biosystems™, Waltham, USA) software and consequently, Beacon Designer [146] was used to ensure no assembly into secondary structures. Finally, to assure specificity for only one (specific) qMSP product, primer sequences were examined with BiSearch Primer Design and Search Tool [147].

3.7. Multiplex quantitative DNA methylation specific PCR

Promoter methylation levels of five genes (*BRCA1$_{me}$, RAD51C$_{me}$, ERCC8$_{me}$, RBBP8$_{me}$,* and *APEX2$_{me}$*) were assessed by multiplex qMSP (Figure 5.), which allows the assessment of multiple genes simultaneously. Primers and TaqMan probes designed specifically for the modified gene sequence, plus fluorochromes and quenchers selected for each probe are listed in Table 2.

Firstly, qMSP reaction for each of the genes was optimised in singleplex separately applying different annealing temperatures (60-64 °C) using several different concentrations of designed F+R primers (0.2, 0.3, or 0.4 μM), 1 μL of bisulfite-treated Human methylated DNA (Zymo Research) and Xpert Fast SYBR (Blue) (GRiSP, Porto, Portugal). Further on, the reactions were optimised in multiplex with TaqMan probes, joining the genes into two panels. The housekeeping gene *β-Actin* was used as an internal reference gene to normalize the assay.

Table 2. Sequences of primers and TaqMan probes with respective fluorochrome and quencher, their final concentration in qMSP reaction mix, and gene distribution in panels for qMSP amplification.

Gene panel	Gene		Sequence (5'-3')	Conc. (μM)	Vendor
1	*β-Actin*$_{me}$	Primers	F - TGGTGATGGAGGAGGTTTAGTAAGT	0.4	Sigma-Aldrich, Steinheim, Germany
			R - ACCAATAAAACCTACTCCTCCCTTAA		
		Probe	Cy5–ACCACCACCCAACACACAATAACAAACACA–QSY	0.1	Applied Biosystems
	BRCA1$_{me}$	Primers	F - GGGAGGCGGTAATGTAAAGATC	0.4	Eurofins Genomics, Ebersberg, Germany
			R - CAATCTTCTTAACGAAAACGCG		
		Probe	FAM-CCCCACAAAAATAACGACAAAACTAACAACG-BHQ1	0.05	Eurofins Genomics, Ebersberg, Germany
	RAD51C$_{me}$	Primers	F - AGTTTCGTGCGGTTAGGTCG	0.4	Eurofins Genomics, Ebersberg, Germany
			R - GTCTTCCCGCGCATCG		
		Probe	Hex-CGTTTTAGCGAGGGCGTGCGG-BHQ	0.05	Eurofins Genomics, Ebersberg, Germany
2	*ERCC8*$_{me}$	Primers	F - AGTAGGGGTAATGTTTTAGTCGTCG	0.4	Eurofins Genomics, Ebersberg, Germany
			R - CCGACCAATAATAAACGCCG		
		Probe	Cy5-AACCGACCTAACTTCTCCGCCTCGA-BHQ2	0.05	Metabion International AG, Planegg, Germany
	RBBP8$_{me}$	Primers	F - AGAAATGTTGTGGCGGTCG	0.4	Eurofins Genomics, Ebersberg, Germany
			R - GCGACGACGCACTTCGTA		
		Probe	Hex-CGGGTAGTTTTCGGTAGTTTCGAGGTAGCG-BHQ1	0.05	Metabion International AG, Planegg, Germany
	APEX2$_{me}$	Primers	F - GTTTCGTTTAGGGTTTCGTCGT	0.4	Eurofins Genomics, Ebersberg, Germany
			R - AAATTTCCAATTAAACTCCCGCT		
		Probe	FAM-CTCGAACAACTAAATCTCAATAC-MGB	0.05	Metabion International AG, Planegg, Germany

Multiplex qMSP assay was carried out in 384-well plates using a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, MA, USA). The multiplex gene combinations used are displayed in Table 2. (referred to as Gene panels).

For each reaction, 1 µL of bisulfite-treated DNA solution, 5 µL of Xpert Fast Probe (GRiSP, Porto, Portugal), a mix with forward and reverse (F+R) primers (0.4 µL of 10 µM F+R per reaction), TaqMan probe (0.05 µL of 10 µM solution per reaction, except 0.1 µL of *β-Actin$_{me}$* probe) to obtain final concentrations, indicated in Table 2., and sterile distilled water (B. Braun, Melsungen, Germany) were added, to a final volume of 10 µL.

The following PCR program was used: 1 cycle at 95ºC for 3 minutes; 40 cycles at 95ºC for 5 seconds and 60ºC for 30 seconds. All samples were run in triplicates, positive control (Human methylated DNA, Zymo Research) was subjected to five serial dilutions (5x factor dilution) and was used to generate a standard curve in each plate (the standards were run in duplicates), allowing for relative quantification and PCR efficiency evaluation. Additionally, two wells of negative controls (Human non-methylated DNA, Zymo Research) and two wells of sterile distilled water were used as negative controls in all plates. All plates displayed efficiency values above 98%. Relative methylation levels were calculated as the ratio between the mean methylation levels of each target gene and the respective value for *β-Actin*, multiplied by 1000.

3.8. Statistical analysis

Statistical analysis methods were applied to perform a pilot study evaluating whether selected DNA repair gene methylation levels could serve as predictive biomarkers or could help to see any tendencies in methylation dynamics related to platinum chemotherapy. *BRCA1$_{me}$*, *RAD51C$_{me}$*, *APEX2$_{me}$*, *RBBP8$_{me}$*, and *ERCC8$_{me}$* levels were compared in different settings, considering diverse cancer models, sample types (pre- or post-chemotherapy), patient response to chemotherapy (poor, partial, or complete), and several platinum-based chemotherapy drugs (CDDP, carboplatin, and oxaliplatin).

Non-parametric tests were performed to compare methylation levels of each gene's promoter between cases and to evaluate associations with clinicopathological features. Mann-Whitney U test was used for comparisons between two groups, while

Wilcoxon signed-rank test was used for comparison of two groups of paired samples (pre-chemo and post-chemo samples of the same patient). A result was considered statistically significant when *p*-value<0.05 (* - p<0.05; ** - p<0.01; *** - p<0.001). The statistical analysis was performed using SPSS 27.0 for Windows software (IBM-SPSS Inc., Armonk, NY, USA). All graphics were assembled using GraphPad Prism 8.0a for Windows software (GraphPad Software, San Diego, CA, USA).

4. RESULTS

4.1. Clinical and pathological data

FFPE tissue samples were obtained from 153 cancer patients, for which 54 paired samples were available (pre-chemotherapy and post-chemotherapy FFPE tissue samples). The group of patients in this study consisted of 76.5% of males and 23.5% of females who received neoadjuvant platinum drug chemotherapy (in the case of TGCT – adjuvant chemotherapy). The most abundant cancer model in this study is SC (41 patients), followed by CRC (30 patients), LC (27 patients), esophageal (26 patients), TGCT (19 patients), and BC (10 patients). Moreover, several cancer models (EC, LC, TGCT) in the study contain patients with different histological types. All of the patients received neoadjuvant platinum-drug chemotherapy but TGCT, which followed adjuvant platinum agent treatment strategy. Considering median age, EC, SC, CRC, BC, and LC patients were treated in a similar age range with medians 62 to 69 years old, whereas TGCT patients were distinct with a much lower median age of 35 years old. Regarding different platinum drugs (CDDP, carboplatin, and oxaliplatin), most of the patients in this study were treated with the same platinum agent depending on the cancer model, while patients with stomach adenocarcinoma were treated with two platinum drugs – CDDP (58.5%) and oxaliplatin (36.6%).

Table 3. Clinical and pathological features of esophageal, stomach, colorectal, bladder, lung, and TGCT cancer patients in the study.

Clinicopathological features	Cancer patients
Number	153
Paired samples	54
	Esophageal cancer
Number (total)	26
Number of patients with paired samples	6
Chemotherapy response (n, %)	
Complete	4/26 (15.4)
Partial	14/26 (53.8)
Poor	8/26 (30.8)
Gender (n, %)	
Male	22/26 (84.6)
Female	4/26 (15.4)
Age (median, interquartile range)	66.5 (61.25-71.5)
Platinum drug (n, %)	
CDDP	2/26 (7.7)
Carboplatin	23/26 (88.5)
Oxaliplatin	1/26 (3.8)
Number of platinum chemotherapy cycles (%)[a]	1 (3.8), 3 (3.8), 4 (30.8), 5 (38.5), 7 (3.8)
Histological subtypes (n, %)	

Adenocarcinoma	6/26 (23.1)
Squamous cell carcinoma	20/26 (76.9)
Clinical stage (n, %)	
I	7/26 (26.9)
II	6/26 (23.1)
III	12/26 (46.2)
IV	1/26 (3.8)
	Stomach cancer
Number (total)	41
Number of patients with paired samples	19
Chemotherapy response (n, %)	
Complete	5/41 (12.2)
Partial	18/41 (43.9)
Poor	18/41 (43.9)
Gender (n, %)	
Male	26/41 (63.4)
Female	15/41 (36.6)
Age (median, interquartile range)	65 (54.75-68)[b]
Platinum drug (n, %)	
CDDP	24/41 (58.5)
Carboplatin	2/41 (4.9)
Oxaliplatin	15/41 (36.6)
Number of platinum chemotherapy cycles (%)[c]	2 (4.9), 4 (26.8), 5 (12.2), 6 (7.3), 8 (4.9)
Histological subtypes (n, %)	
Adenocarcinoma	41/41 (100)
Clinical stage (n, %)	
I	6/41 (14.6)
II	14/41 (34.2)
III	11/41 (26.8)
IV	10/41 (24.4)
	Colorectal cancer
Number (total)	30
Number of patients with paired samples	12
Chemotherapy response (n, %)	
Complete	1/30 (3.3)
Partial	18/30 (60)
Poor	11/30 (36.7)
Gender (n, %)	
Male	20/30 (66.7)
Female	10/30 (33.3)
Age (median, interquartile range)	62 (52.5-65)[d]
Platinum drug (n, %)	
Oxaliplatin	30/30 (100)
Number of platinum chemotherapy cycles (%)[e]	2 (10), 3 (6.7), 4 (3.3), 5 (6.7), 6 (6.7), 7 (3.3), 9 (3.3), 12 (10)
Histological subtypes (n, %)	
Adenocarcinoma	30/30 (100)
Clinical stage (n, %)	
I	3/30 (10)
II	2/30 (6.7)

III	6/30 (20)
IV	19/30 (63.3)
	Bladder cancer
Number (total)	10
Number of patients with paired samples	1
Chemotherapy response (n, %)	
Complete	1/10 (10)
Partial	4/10 (40)
Poor	5/10 (50)
Gender (n, %)	
Male	9/10 (90)
Female	1/10 (10)
Age (median, interquartile range)	69 (63-72)[f]
Platinum drug (n, %)	
Cisplatin	10/10 (100)
Number of platinum chemotherapy cycles (%)[g]	4 (40)
Histological subtypes (n, %)	
Urothelial carcinoma	10/10 (100)
Clinical stage (n, %)	
0	1/10 (10)
II	7/10 (70)
III	2/10 (20)
	Lung cancer
Number (total)	27
Number of patients with paired samples	16
Chemotherapy response (n, %)	
Complete	2/27 (7.4)
Partial	11/27 (40.7)
Poor	14/27 (51.9)
Gender (n, %)	
Male	21/27 (77.8)
Female	6/27 (22.2)
Age (median, interquartile range)	62 (56-68.5)
Platinum drug (n, %)	
CDDP	26/27 (96.3)
Carboplatin	1/27 (3.7)
Number of platinum chemotherapy cycles (%)[h]	3 (11.1), 4 (29.6), 5 (3.7), 6 (7.4)
Histological subtypes (n, %)	
Adenocarcinoma	18/27 (66.7)
Squamous cell (non-small cell) carcinoma	6/27 (22.2)
Mixed (adenosquamous carcinoma)	1/27 (3.7)
Large cell neuroendocrine carcinoma	1/27 (3.7)
Small cell carcinoma (mixed)	1/27 (3.7)
Clinical stage (n, %)	
I	3/27 (11.1)
II	8/27 (29.6)
III	13/27 (48.1)
IV	3/27 (11.1)
	TGCT
Number (total)	19

Number of patients with paired samples	-
Chemotherapy response (n, %)	
Partial	9/19 (47.4)
Poor	10/19 (52.6)
Gender (n, %)	
Male	19/19 (100)
Age (median, interquartile range)	33 (25-35)
Platinum drug (n, %)	
CDDP	18/19 (94.7)
Carboplatin	1/19 (5.3)
Number of platinum chemotherapy cycles (%)[i]	2 (5.3), 3 (52.6), 4 (31.6), 6 (5.3)
Histological subtypes (n, %)	
Seminoma	9/19 (47.4)
Non-seminoma	10/19 (52.6)
Clinical stage (n, %)[j]	
I	4/19 (21.1)
II	7/19 (36.8)
III	7/19 (36.8)

[a] – 5 cases missing number of chemo cycles data; [b] – 4 cases missing chemotherapy treatment beginning date; [c] – 18 cases missing number of chemo cycles data; [d] – 7 cases missing chemotherapy treatment beginning date; [e] – 15 cases missing number of chemo cycles data; [f] – 1 case missing birthdate; [g] – 6 cases missing number of chemo cycles data; [h] – 13 cases missing number of chemo cycles data; [i] – 1 case missing number of chemo cycles data; [j] – 1 case missing stage data.

4.1. Selection of DNA repair genes and CpG sites of interest for the study

Preliminary *in silico* analysis was performed to identify DNA repair genes for further study and CpG sites within the promoter were chosen for further design of qMSP primers and probes. TCGA database for main solid cancers treated with platinum drugs was used and an algorithm was applied to select the primary set of genes for further analysis (Figure 6.). Moving on, the genes were filtered for the ones that contained CpGs in the promoter region, and finally, they were subjected to correlation analysis (promoter methylation and expression), resulting in a list of 74 genes (Figure 6.). Further on, CpGs in the promoter regions of the genes were analysed separately in order to select the DNA repair genes with as many as possible overlapping CpGs of interest in several cancer models to follow the pan-cancer setting.

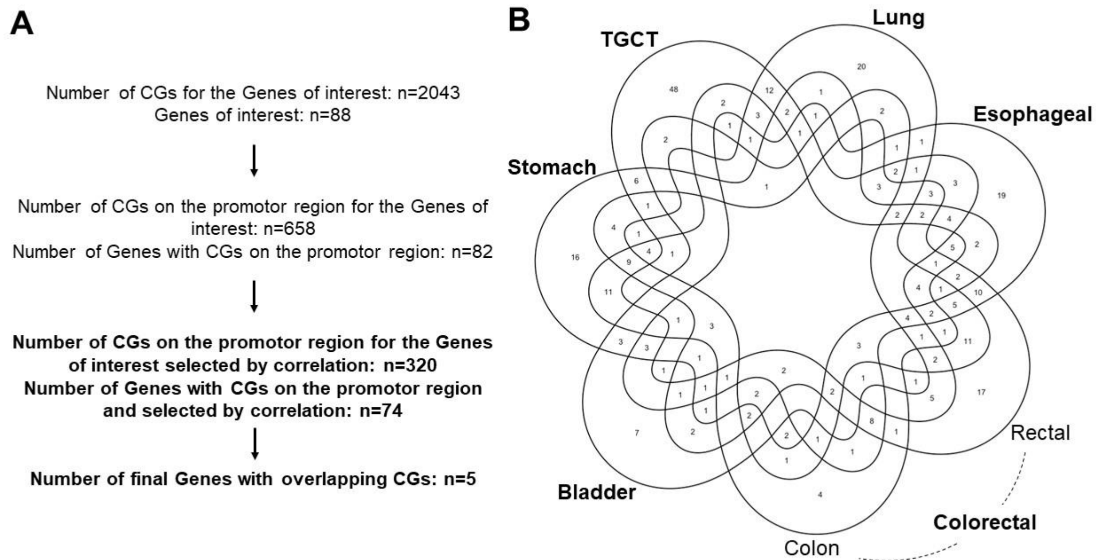
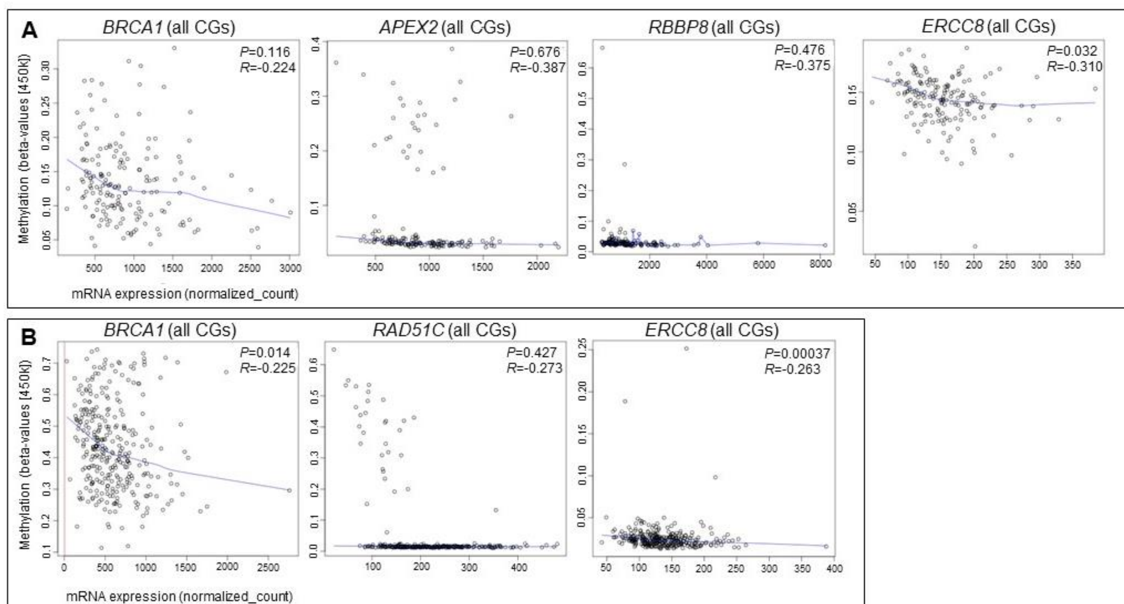


Figure 6. The sequence of steps for selection of DNA repair genes from TCGA for the study (A). Veen diagram showing the number of CpGs shared by different cancer types (B).

Finally, five DNA repair genes whose promoter methylation was observed to be inversely correlated to gene expression in cancer patients were chosen for the study – *BRCA1*, *RAD51C*, *APEX2*, *RBBP8*, *ERCC8* contained specific CpG sites with pronounced expression anti-correlation. Preliminary graphs indicating the correlation of expression and methylation *in silico* were assembled for each gene (Figure 7.).



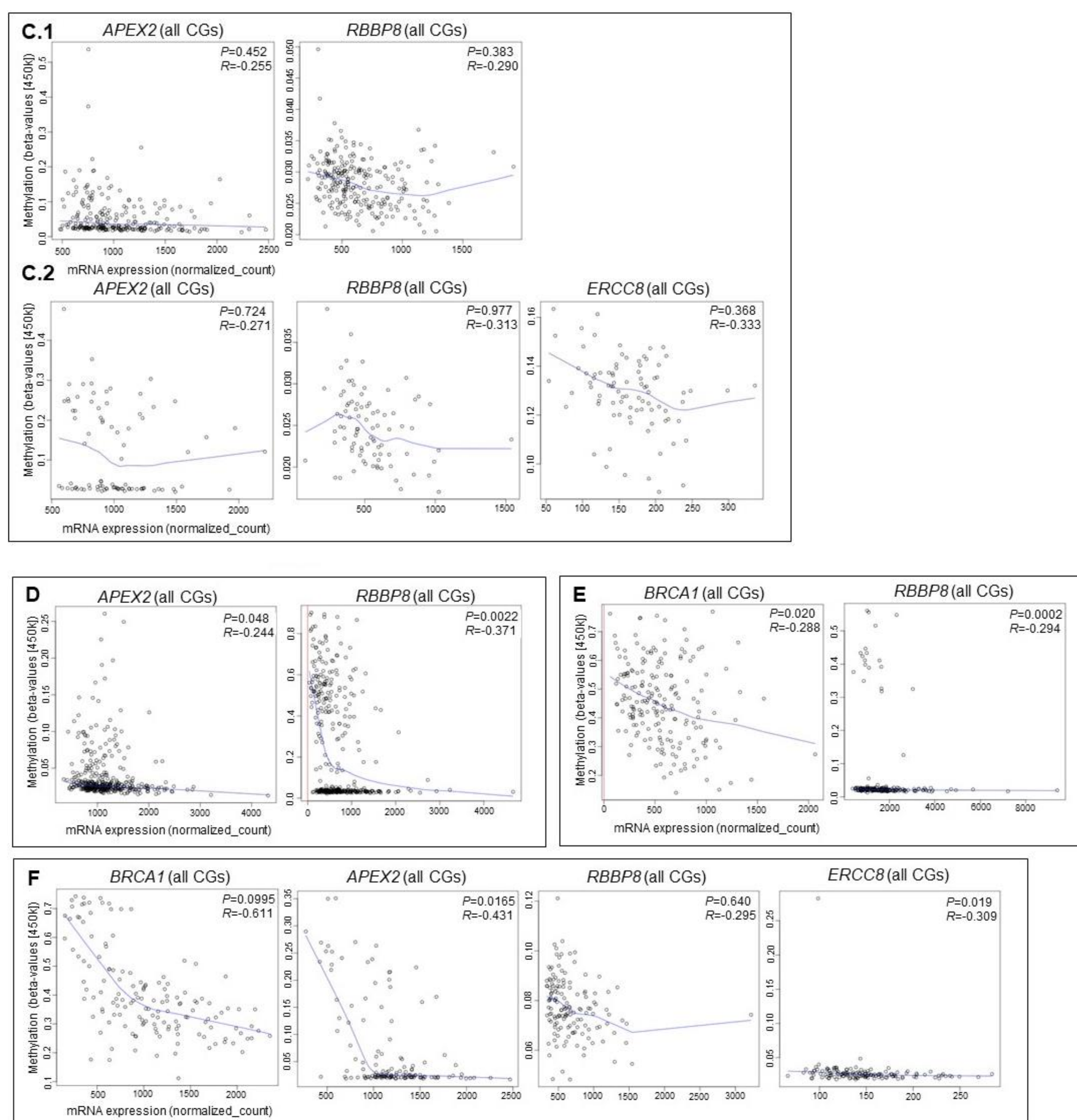


Figure 7. Graphs of methylation vs gene expression for each gene in esophageal cancer (A), stomach (B), colon (C.1) and rectal (C.2), bladder (D), lung (E) cancers, and TGCT (F). Each point in the graph represents a separate patient indicating the mean methylation levels of all CGs. Blue line – locally weighted scatterplot smoothing. Separate graphs for each relevant CG site is provided in Appendix II: Supplementary Figure 1.

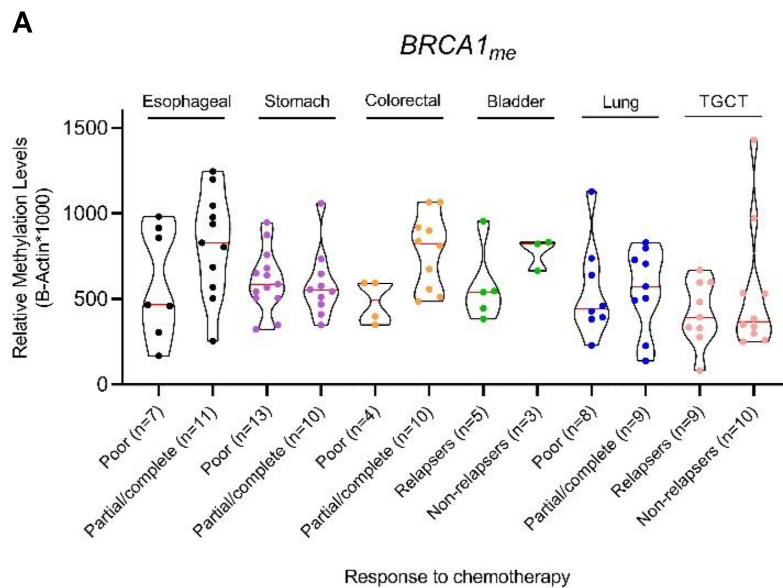
4.2. Methylation levels of selected DNA repair gene promoters in patient tissue samples

After the DNA repair genes of interest were selected, primers were designed and tested separately for each gene in singleplex reactions using positive control of human methylated DNA as a template to ensure optimal amplification of specific products only. Afterwards, qMSP reactions for evaluation of DNA repair gene methylation levels were

optimised in multiplex settings, and finally, all the purified and bisulfite-treated DNA samples were tested and the obtained results were normalized.

4.2.1. Pre-chemo methylation levels of DNA repair genes in tumor biopsy specimens

Since the main goal was to evaluate whether methylation of selected gene promoters could serve as predictive biomarkers, only pre-chemotherapy samples were compared for methylation differences between themselves separating into groups of poor and partial/complete responders (relapsers and non-relapsers in BC and TGCT). A representative number of cases showed detectable methylation levels for *BRCA1* and *APEX2* genes (Figure 8.), while very low methylation levels to the absence of methylation were found for the remaining genes (*RAD51C$_{me}$*, *RBBP8$_{me}$*, *ERCC8$_{me}$* graphs in Appendix II: Supplementary Figure 2.). Consequently, *RAD51C$_{me}$*, *RBBP8$_{me}$*, and *ERCC8$_{me}$* gene methylation levels did not differ between the groups of poor and partial/complete responders (relapsers and non-relapsers in BC and TGCT) in samples of any cancer model studied. Considering *BRCA1$_{me}$*, patients with a better response to chemotherapy disclosed a tendency of higher relative methylation levels while in the case of *APEX2$_{me}$* responders showed an opposite tendency.



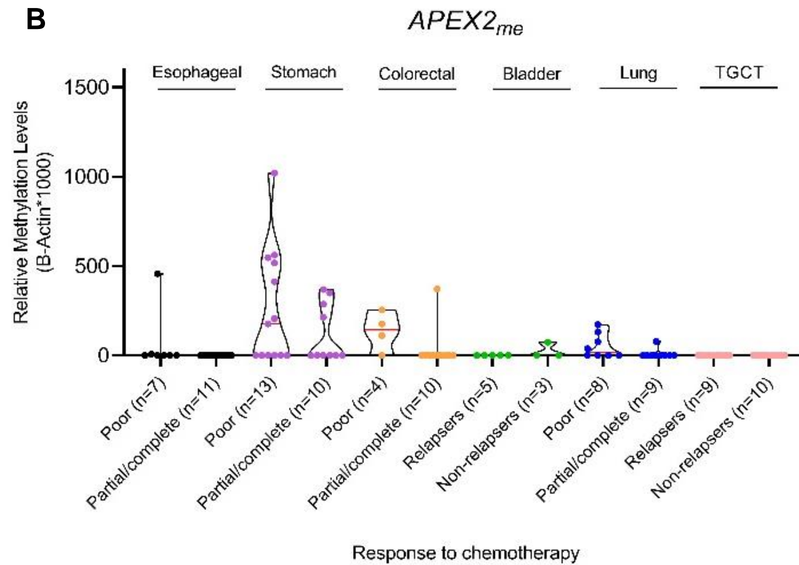


Figure 8. Distribution of *BRCA1* (A) and *APEX2* (B) relative methylation levels in pre-chemotherapy samples of esophageal, stomach, colorectal, bladder, lung, and TGCT patients, with regards to chemotherapy response. Mann-Whitney U Test between poor and partial/complete (relapsers and non-relapsers in BC and TGCT). Red horizontal lines represent median methylation levels, n indicates the number of patient samples.

Indeed, *BRCA1* was a unique DNA repair gene in terms of detectable methylation levels. When all the pre-chemotherapy samples of studied cancer models were lumped, *BRCA1* methylation levels were significantly higher (p=0.034) in patients that showed partial/complete response to chemotherapy (Figure 9.).

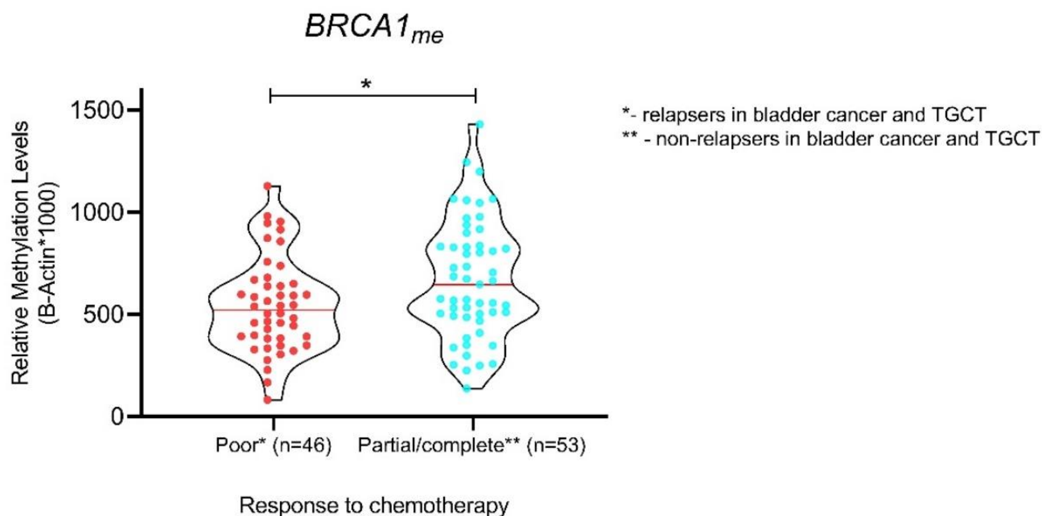


Figure 9. Distribution of *BRCA1* relative methylation levels in pre-chemotherapy samples of esophageal, stomach, colorectal, bladder, lung, and TGCT patients joined together, with regards to chemotherapy response. Mann-Whitney U Test between poor and partial/complete (relapsers and non-relapsers in BC and TGCT). Red horizontal lines represent median methylation levels, n indicates the number of patient samples. * - p<0.05.

4.2.2. Pre- and post-chemo methylation dynamics of DNA repair genes in tumor tissues

Aiming to evaluate how platinum chemotherapy treatment affected DNA repair gene methylation levels, pre-chemotherapy tumor biopsy samples were compared to post-chemotherapy tumor tissue surgical specimens (Figure 10). BC model was not included in the analysis due to a low number of available samples and TGCT was also omitted from pre- and post-chemo analysis because only pre-chemotherapy samples were included in the initial patient cohort. Overall, most of the cancer models (SC, CRC, and LC) displayed a tendency of increased relative methylation levels after chemotherapy, except EC patients most likely due to a reduced number of samples. SC and LC patients showed significantly higher *BRCA1* methylation levels in post-chemotherapy samples compared with pre-chemotherapy samples (p=0.018 and p=0.007 accordingly).

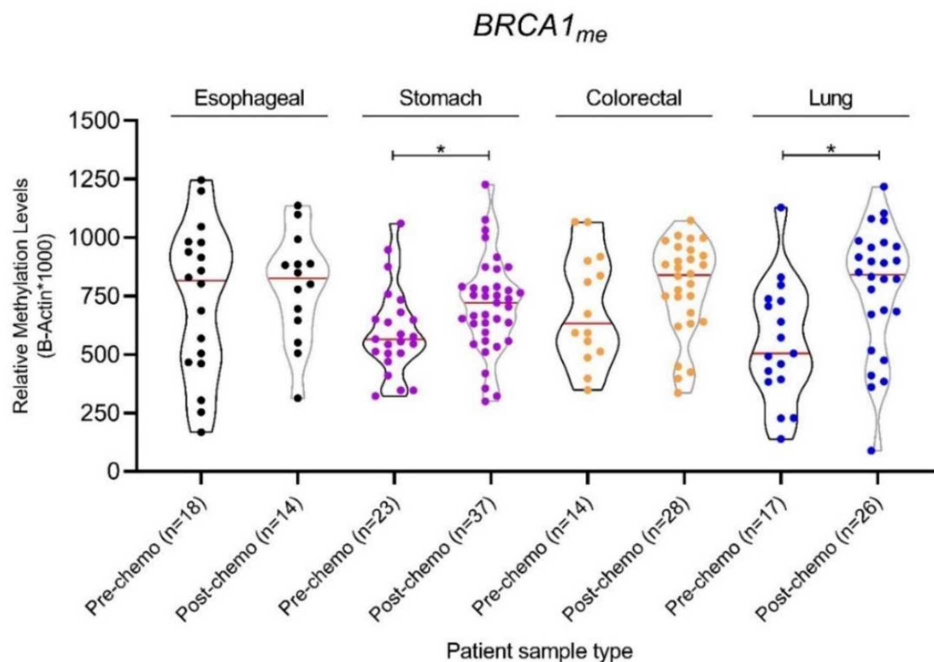


Figure 10. Distribution of *BRCA1* relative methylation levels in pre- and post-chemotherapy samples (paired and non-paired) of esophageal, stomach, colorectal, and lung patients. Mann-Whitney U Test between pre- and post-chemo. Red horizontal lines represent median methylation levels, n indicates the number of patient samples. * - p<0.05.

Considering the dynamics of relative methylation levels in patients whose pre- and post-chemotherapy FFPE samples were available, SC and LC were the cancer models for which most paired samples were obtained and the methylation dynamics are

represented in Figure 11. Poor and partial/complete chemotherapy responders showed a tendency of increasing methylation levels in post-chemotherapy samples. LC patients displayed significant differences before and after the treatment in poor (p=0.017) and partial/complete responders (p=0.036). Interestingly, a similar trend of increasing methylation levels was also found for other tumor models like EC and CRC although the number of paired cases was small (Appendix II: Supplementary Figure 3.). Additionally, *APEX2*$_{me}$ level dynamics were also observed in LC and SC, though in this cohort the number of paired cases with traceable methylation levels of *APEX2* was low (Appendix II: Supplementary Figure 4.). However, the data indicated a similar tendency of increasing DNA repair gene promoter methylation levels after platinum chemotherapy.

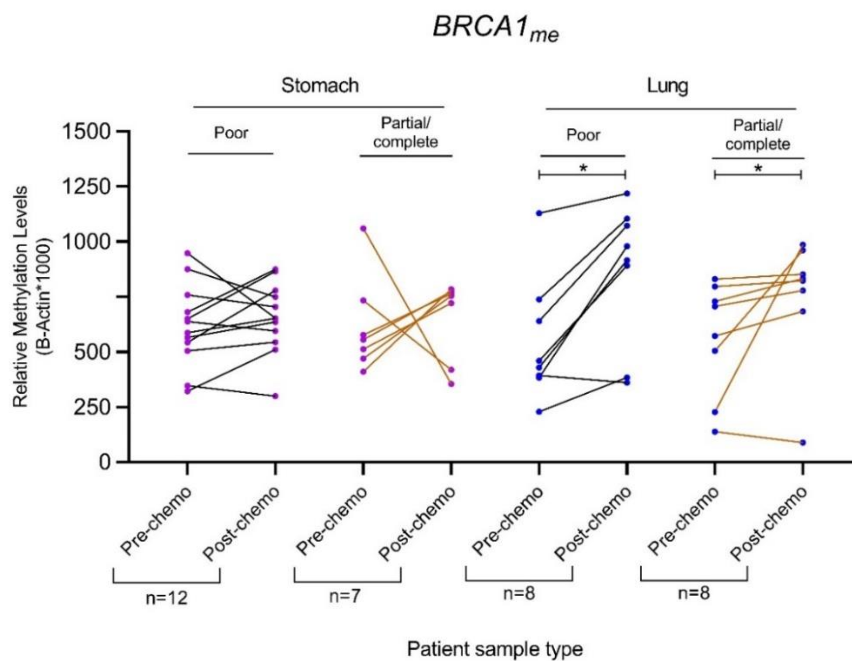


Figure 11. Distribution of *BRCA1* relative methylation levels in pre- and post-chemotherapy samples (paired) of stomach and lung cancer patients, regarding platinum chemotherapy response. Wilcoxon signed-rank Test between pre- and post-chemo. Red horizontal lines represent median methylation levels, n indicates the number of patients. * - p<0.05.

Moreover, when all patient pre- and post-chemotherapy paired samples were lumped, a significant increase (p<0.001) in promoter relative methylation levels can be observed (Figure 12). This phenomenon is valid for all cancer models independently of patients' chemotherapy response.

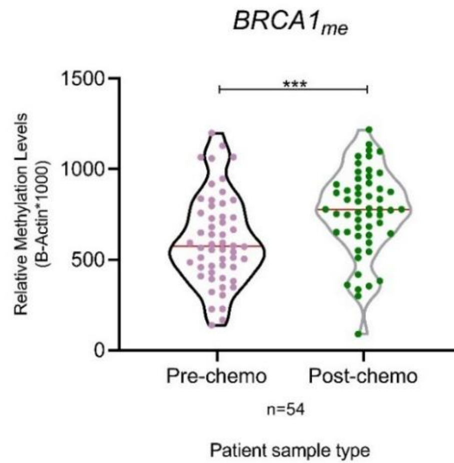


Figure 12. Distribution of *BRCA1* relative methylation levels in pre- and post-chemotherapy samples (paired) of all cancer patients included in the study (esophageal, stomach, colorectal, bladder, and lung cancer). Wilcoxon signed-rank Test between pre- and post-chemo. Red horizontal lines represent median methylation levels, n indicates the number of patients. *** - $p<0.001$.

Finally, since the studied patients were treated with three different platinum drugs (CDDP, carboplatin, and oxaliplatin), we tested whether the same effect was observed on DNA repair genes methylation levels (Figure 13). In order to conduct this analysis, patients were categorised into three groups depending on the platinum drug received and, in these groups, patient pre- and post-chemotherapy sample methylation levels were compared. The same tendency of increased methylation levels in post-chemotherapy samples compared to pre-chemotherapy was observed in patients treated with any type of platinum drug. Statistical significance was achieved for CDDP (p<0.001).

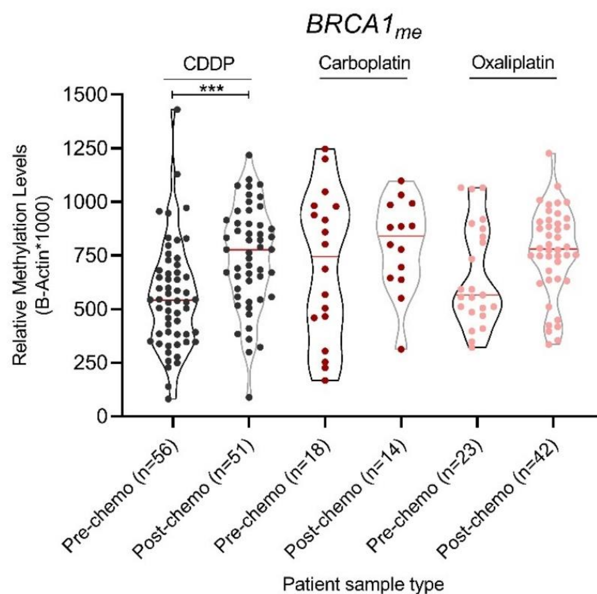


Figure 13. Distribution of *BRCA1* relative methylation levels in pre- and post-chemotherapy samples of all cancer models under study (esophageal, stomach, colorectal, bladder, and lung) patients (paired and non-paired samples) with regards

to the platinum agent received (CDDP, carboplatin, oxaliplatin). Mann-Whitney U Test between pre- and post-chemotherapy. Red horizontal lines represent median methylation levels, n indicates the number of patient samples.

4. DISCUSSION

Platinum agents (CDDP, carboplatin, and oxaliplatin) are commonly used drugs due to their beneficial therapeutic effects in a spectrum of different cancer models [148]. However, there are drawbacks related to the application of platinum-based drugs – some patients manifest primary or acquired resistance to platinum chemotherapy during the treatment course and experience severe side effects [149]. This scenario might be avoided if predictive biomarkers could be detectable in liquid biopsies, thus would help rapidly and reliably identify the patients that may benefit from platinum-based treatment [96, 150]. Currently, there are a plethora of studies directed to the establishment of such predictive epigenetic biomarkers related to platinum drug resistance [97, 102, 105, 111] yet due to suboptimal predictive value, they are still not used in clinics. DNA repair genes are important molecular components implicated in platinum-related DNA damage repair in neoplastic cells and thus resistance to platinum [151]. Consequently, we performed a pilot study aiming to evaluate whether it is feasible to establish a DNA repair gene methylation biomarker for platinum chemotherapy response prediction by studying patients' tumor tissues. Additionally, we evaluated DNA repair gene methylation dynamics before and after the chemotherapy to evaluate the possible application of epidrugs.

The rationale of the study was to identify hypomethylated DNA repair genes in pre-chemotherapy samples of poor responders (relapsers in TGCT and BC), as the cells are capable to resolve platinum-caused DNA damage with increased expression of DNA repair gene(s), while in good responders (non-relapsers in TGCT and BC) DNA repair genes would be hypermethylated and have an impact on death of neoplastic cells due to reduced DNA repair capacity. First of all, *in silico* analysis was used to interrogate the selection of candidate DNA repair genes and their CpG sites of interest, a similar selection approach as previously published data by our research team [12]. *BRCA1* is one of the so-called guardians of the genome [152] due to its crucial role in maintaining genomic stability, tumor suppressor role, having a central function of facilitating cellular responses to DNA damage, and participating in double-strand DNA damage repair by HR [153, 154]. Even though mutations of *BRCA1* are well-known to be related to breast cancer, as they impair the correct function of this enzyme enabling carcinogenesis pathways, the defective enzyme version participates in neoplastic progression in a variety of other cancers [155, 156]. In addition, *BRCA1* down-regulation as a consequence of mutations [157] or promoter methylation [12] is already known to serve as a biomarker of PARP inhibitor sensitivity. The main goal of this study was to establish a biomarker that would be predictive of the platinum-based chemotherapy response.

Nonetheless, although the study included a reduced number of pre-chemotherapy samples (99 in total), *BRCA1*$_{me}$ emerged as a possible candidate for platinum resistance biomarker, since a tendency of different methylation levels was observed in patients who respond poorly or benefit from the treatment prior to chemotherapy. Low number of samples was a limitation for establishing a relative methylation level threshold between responders and non-responders for assessing its potential as predictive biomarker.

Following *in silico BRCA1*$_{me}$ results, the promoter was determined to have relevant CpG sites in 4 out of 6 cancer models – EC, SC, LC, TGCT and relatively high methylation levels in all except EC cases. Interestingly, in FFPE samples, *BRCA1* showed high methylation levels in all of the cancer models, contrary to the rest of the genes in the study. *BRCA1* expression is known to be regulated by promoter methylation not only in EC [158], SC [159], LC [160], and TGCT [12] but also in BC [161], while little is known about CRC [162]. The reason behind methylation levels being detected in all of the cases could be due to *BRCA1* being a crucial TSG that is abundantly expressed in most of the tissues [163] or could be related to well-optimised *BRCA1*$_{me}$ qMSP conditions which allow highly sensitive methylation detection. In addition, one of the drawbacks was the lack of comparison of cancer patient methylation levels with normal controls during the stage of *in silico* analysis. This way, it would be possible to compare *BRCA1*$_{me}$ levels between cancer patients and controls to confirm whether the methylation levels observed were related to intrinsic resistance or they change with the carcinogenesis process.

In case *BRCA1*$_{me}$ results would be confirmed in a larger number of pre-chemotherapy samples, a clinically useful predictive biomarker would be established for one or several cancer models because we found that pre-treatment methylation status could be predictive of responsiveness to chemotherapy. Further on, the biomarker performance would be evaluated in patient plasma samples and *BRCA1*$_{me}$ could be included in a gene panel to be applied for examination of cancer patients before neoadjuvant chemotherapy. There is a high clinical utility of such a biomarker because, in the case of predicted good platinum-agent chemotherapy responders, reduced drug concentration could be applied, knowing the high possibility of enhanced chemotherapy response. Apart from resistance to the treatment, platinum drugs are well-known for high toxicity (e.g. nephrotoxicity, ototoxicity, and peripheral neurotoxicity) [20], side effects, and residual drug concentration in the blood even up to 20 years after the treatment [164, 165] which could be reduced in case medical oncologist would be confident in applying lower drug concentration. In addition, a predictive biomarker would be beneficial for

surgeons to know upfront patient response because it could make them assertive of good tumor resection reducing intervention into nearby healthy tissues.

Considering the remaining DNA repair genes in this study, no apparent relevant clinical value was observed. *RAD51C* is also a DNA repair and TSG whose enzyme product is implicated in complexes that take part in HR [166]. Even though *RAD51C* precise function in neoplasia formation and treatment resistance has been obscure until recently, its' methylation has emerged as a biomarker of PARP inhibitor resistance [167, 168]. Similarly, *RBBP8* gene is also involved in the HR pathway [169] though it has been determined to be regulated by methylation almost only in BC [170]. *ERCC8* is a substrate-recognition component in the CSA complex, participating in transcription-coupled nucleotide excision repair and also involved in double-strand break repair by NHEJ [171]. Currently, there is no information about *ERCC8* methylation in the context of any cancer model. Finally, *APEX2* gene is unique compared to the rest of the genes, since it seems to be involved in BER which patches up DNA apurinic/apyrimidinic sites with single-strand breaks [121]. Since platinum drugs cause the most damage by creating dsDNA breaks [118], a BER protein selected in this context could indicate new insights into platinum drug mechanism of action (e.g., in the stomach, and lung where *APEX2* amplifications were observed). Moreover, *APEX2* was only found to be hypomethylated in head and neck squamous cell carcinoma cases [172].

Regarding pre-chemotherapy samples, *APEX2* showed methylation in several cancers (SC, CRC, LC) though with lower levels than *BRCA1*. Considering *in silico* analysis, *APEX2* was predicted to have relevant CGs in EC, CRC, BC, and TGCT, and in the results, the gene had a significant anti-correlation between methylation and expression in BC and TGCT. However, in primary samples, the methylation levels were quite disparate among different tumors, possibly due to the fact that our cohort is reduced, not adequately reflecting $APEX2_{me}$ profiling. Additionally, the reduced frequency of methylation could be impacted by low purified DNA levels due to the detrimental effects of formalin fixation on nucleic acids [173]. Importantly, DNA concentration measurement might also have effects on the successful method workflow. Low concentrations of DNA purified from FFPE samples quantification values obtained with Nanodrop™ should be compared to Qubit™ since there are studies suggesting that Qubit™ is more accurate due to lower measurement error (1%) than Nanodrop™ (5%) [174]. Interestingly, despite the reduced number of patient samples, $APEX2_{me}$ levels followed a different tendency than $BRCA1_{me}$, considering poor and partial/complete responders (relapsers and non-relapsers in TCGT and BC). Methylation levels were

lower in SC, CRC, and LC partial/complete than in poor responders, meaning higher *APEX2* expression could be related to better platinum chemotherapy response in a certain group of patients which should be confirmed in a bigger cohort. However, these tendencies can only be proven if a high number of cases could be tested.

The rest of the genes – $ERCC8_{me}$, $RBBP8_{me}$, $RAD51C_{me}$ – almost did not amplify or presented extremely low methylation levels. It can be originated from the fact that not all of the chosen DNA repair genes showed relevant CGs in all cancer models (e.g., in LC only $BRCA1_{me}$ and $RBBP8_{me}$ were selected) and the *in silico* analysis revealed reduced methylation levels. The qMSP results support *in silico* data for these genes ($APEX2_{me}$, $ERCC8_{me}$, $RBBP8_{me}$, $RAD51C_{me}$), meaning our cohort was too small and the qMSP could also be lacking sensitivity to detect such low methylation levels. In order to see whether the DNA repair gene expression is regulated by methylation in patients, a more extensive analysis including a higher number of patients should be executed and the results should be compared with healthy controls.

The second objective of this study was to compare DNA repair gene methylation dynamics before and after platinum chemotherapy. Firstly, lumped samples (paired and non-paired) for separate cancer models were compared. SC and LC showed significantly higher $BRCA1_{me}$ levels after chemotherapy, whereas only a similar tendency was observed in CRC and no significant change was found in EC samples. In SC and LC (cancer models in the study with most paired patient samples), paired samples (when pre- and post-chemo samples of the same patient are available) increased methylation levels were observed in the majority of samples although without statistical significance. Further on, in case the patients were grouped regarding the platinum drug received for chemotherapy treatment, the same pattern of increasing $BRCA1_{me}$ in post-chemotherapy samples was also observed. Overall, the most significant results were obtained when all paired samples were lumped not taking treatment response into account and comparing $BRCA1_{me}$, which reflects the common rule that no matter the treatment response or platinum agent applied, $BRCA1_{me}$ increased in post-chemotherapy samples in cancer models included in the study. Taking the rest of the DNA repair genes into account, only *APEX2* was observed to present some methylation dynamics, also following the tendency of increasing methylation levels after chemotherapy. The rest of the genes ($RAD51C_{me}$, $ERCC8_{me}$, $RBBP8_{me}$) did not amplify.

The results obtained in this work indicate that without regard to chemotherapy response – poor or partial/complete – and without difference in which platinum drug is used, $BRCA1_{me}$ levels were increased. In partial/complete responders, the increase of

methylation was expected, following the rationale of this study, meaning a reduced expression of DNA repair genes may lead to more DNA damage in neoplastic cells which cannot be fixed, thus leading to cells apoptosis. Indeed, increased *BRCA1* methylation was already known to predict sensitivity to platinum agents in breast and ovarian cancers [175]. Contrarily, increased methylation levels were also observed in poor responders, which in theory could be related to the mechanism that reduced *BRCA1* expression is associated with less DNA damage response and lack of *BRCA1* reduces the induction of apoptotic pathways which sustain neoplastic cell survival independently of the platinum chemotherapy effects on DNA. It was shown by others that *BRCA1*-deficient cells are defective in the arrest of DNA synthesis (cell cycle phase S) after damage with ionizing radiation [176]. Hence, increased methylation levels during platinum chemotherapy may indicate the emergence of acquired resistance to the treatment. In this scenario, epigenetic drugs may be applied to recover *BRCA1* expression and sensitize neoplastic cells for further platinum chemotherapy or other consequent treatment [138]. Moreover, it is possible that changes in our selected DNA repair gene methylation levels during platinum chemotherapy may be a passive phenomenon, not necessarily related to the treatment applied.

Interestingly, a study performed with breast cancer patient samples showed that there were differences in methylation levels in responders and non-responders. The two patient groups had different CpG methylation and shared just 2.2% of the same CpGs in the *BRD9* gene, having the most differentially methylated CpG sites in promoter CpG-islands for responders and the so-called open-sea region for non-responders [177]. Further analysis of our DNA repair genes should be performed to see whether the same CpG sites are relevant for poor and partial/complete responders in the case of our study. In addition, a cohort with a higher number of samples is necessary to confirm whether the results obtained could be related to any of the clinicopathological factors, like a specific methylation pattern depending on the histopathological classification of tumors or clinical stages, metastasized or non-metastasized patient cases, etc. Further on, *in vitro* testing of the effect of cisplatin-derived drugs, would allow assessing $BRCA1_{me}$ dynamics and cellular apoptosis in real-time. Finally, if proven, $BRCA1_{me}$ could be assessed in patients' plasma samples using techniques like ddPCR to increase detection sensitivity, which would be amenable to be translated to the clinical setting enabling the determination of platinum drug resistance before patients' treatment.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our results support the hypothesis that DNA repair genes have the potential to be used as predictive biomarkers to foresee patient response to platinum-based chemotherapy, which could reduce chemotherapy side effects and also avoid the usage of platinum-based agents in patients with intrinsic resistance. Moreover, our results indicate that comparison of pre- and post-chemotherapy methylation levels of DNA repair gene may aid in the evaluation of acquired resistance to chemotherapy and to identify which patients would benefit from epidrugs.

Considering our findings, we foresee to:

- Repeat *in silico* analysis including healthy controls to select additional DNA repair genes with high methylation levels;
- Re-optimise DNA purification steps, concentration measurement, and qMSP reaction;
- Perform *in vitro* experiments with cell lines, to investigate platinum drug effects on DNA repair gene methylation levels and cell death;
- Expand patient cohort and determine if methylation levels are related to the clinicopathological parameters;
- Determine epigenetic biomarkers' accuracy to predict patients' response to platinum-based agents.

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8. APPENDIX

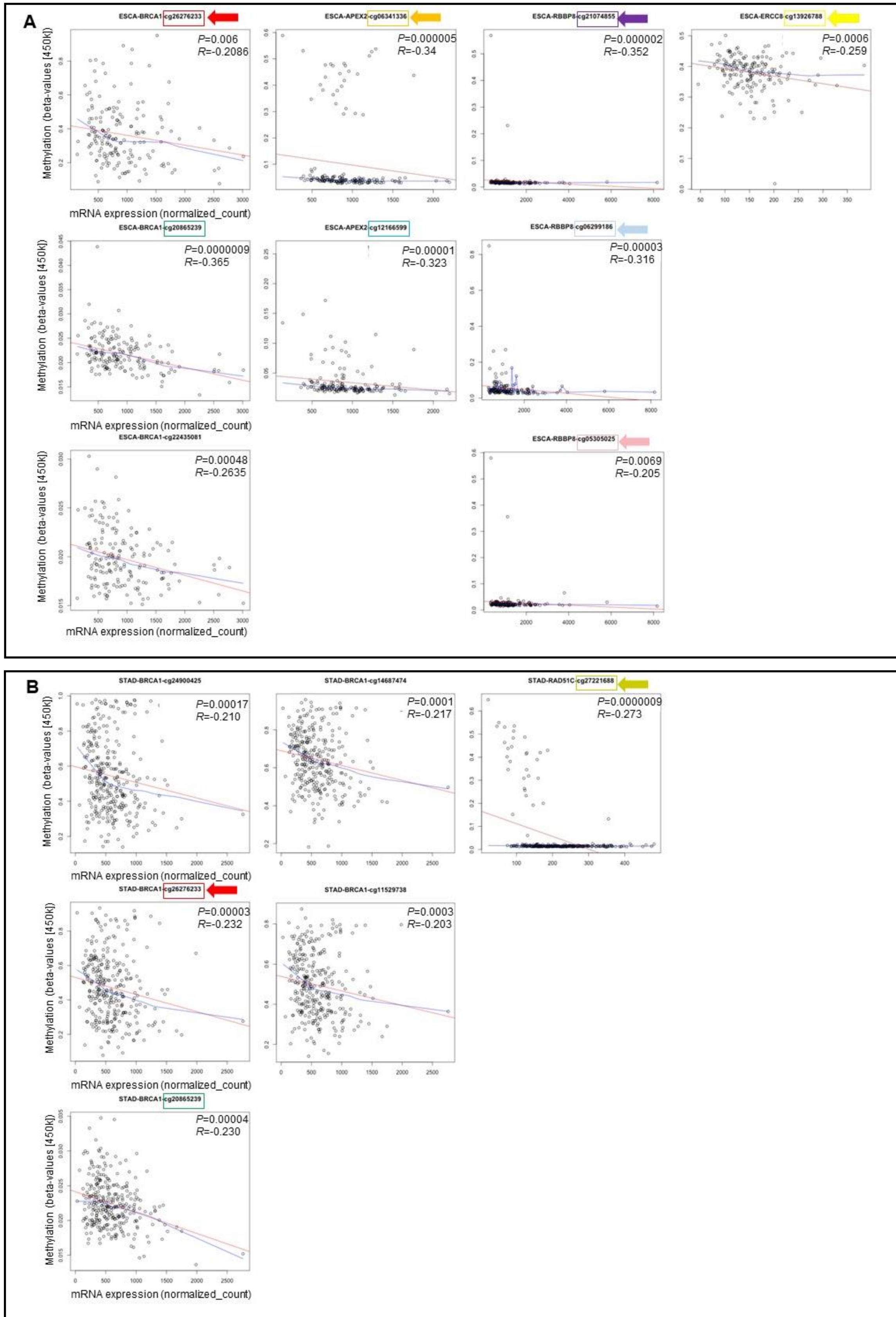
8.1. Appendix I: Clinical database construction

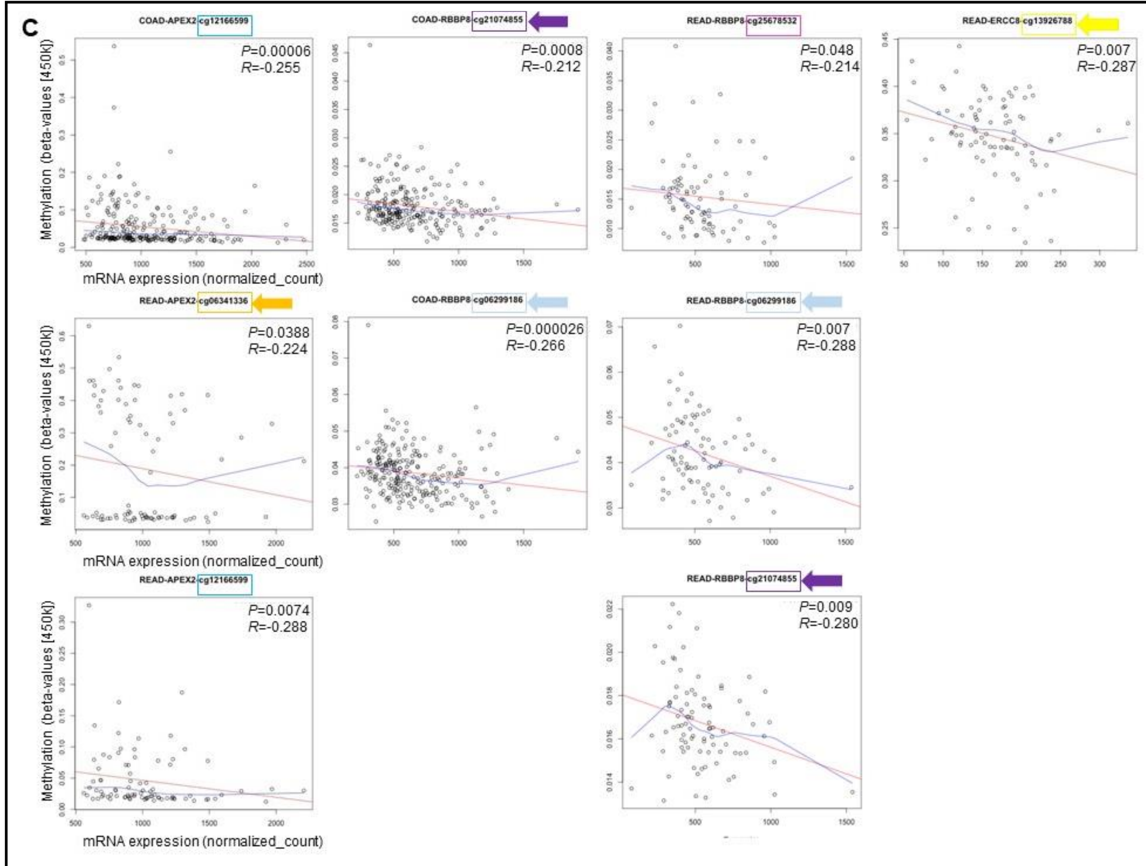
In order to see whether selected DNA repair gene methylation levels correlated with any of available clinical data regarding the patient cohort, clinical information was collected and displayed in a database. This information was obtained from IPO patient database.

Firstly, basic information was collected: gender, birthdate, age at diagnosis, date of surgery, date of the last follow-up, treatment strategy (combination of drugs), number of platinum chemotherapy cycles (neoadjuvant chemotherapy for esophageal, stomach, colorectal, bladder, lung and adjuvant for TGCT), response to chemotherapy, date of the beginning of the treatment, date of the last day of treatment, date of surgery, the current state (if patient died). Since some of the relevant patient FFPE tumor tissue samples were located in other institutions, the samples were requested in order to have the maximum possible number of paired (pre- and post-chemotherapy) patient samples.

Further on, remaining information about the tumor was retrieved from the database. In these were included: clinical stage (TNM) and histological subtype.

8.2. Appendix II

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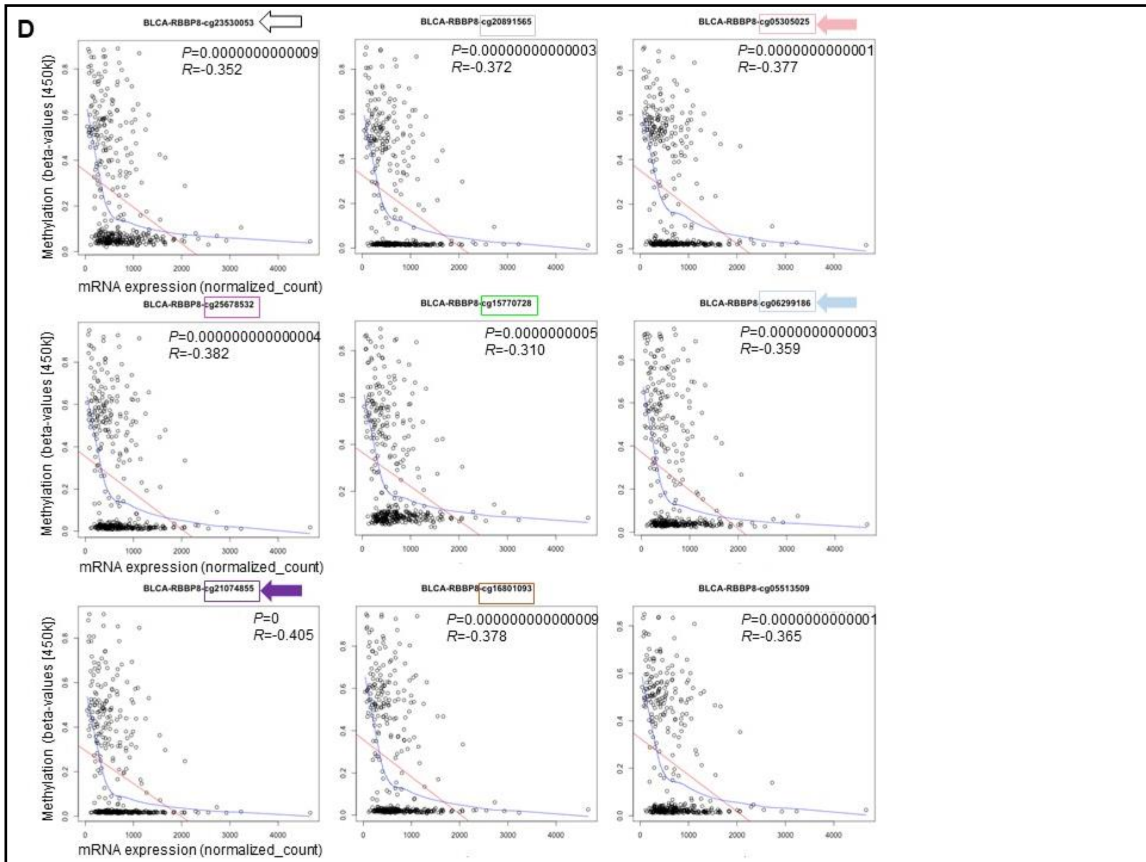
Methylation (beta-values [450k])
mRNA expression (normalized_count)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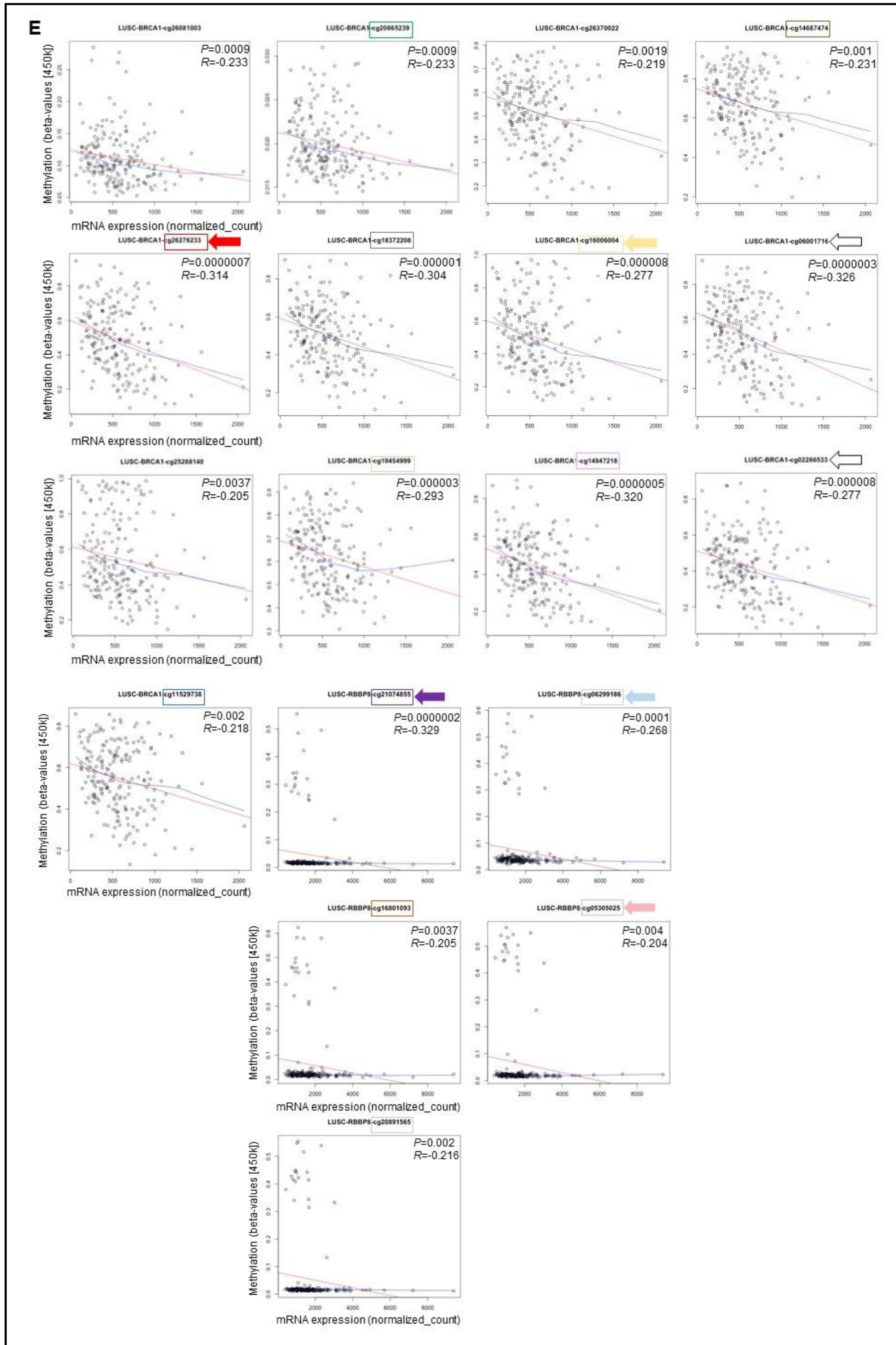
R=-0.365
Methylation (beta-values [450k])
mRNA expression (normalized_count)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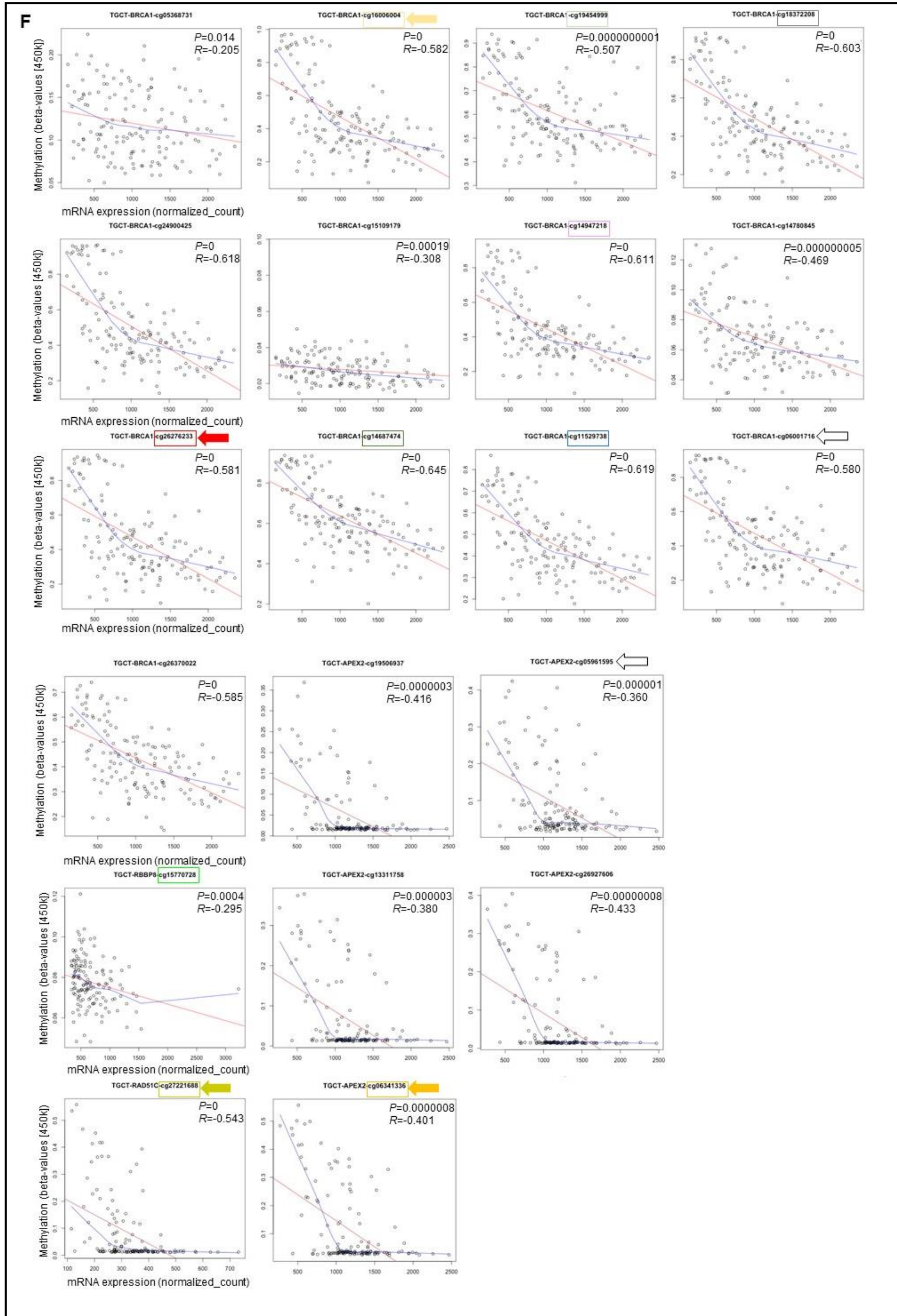
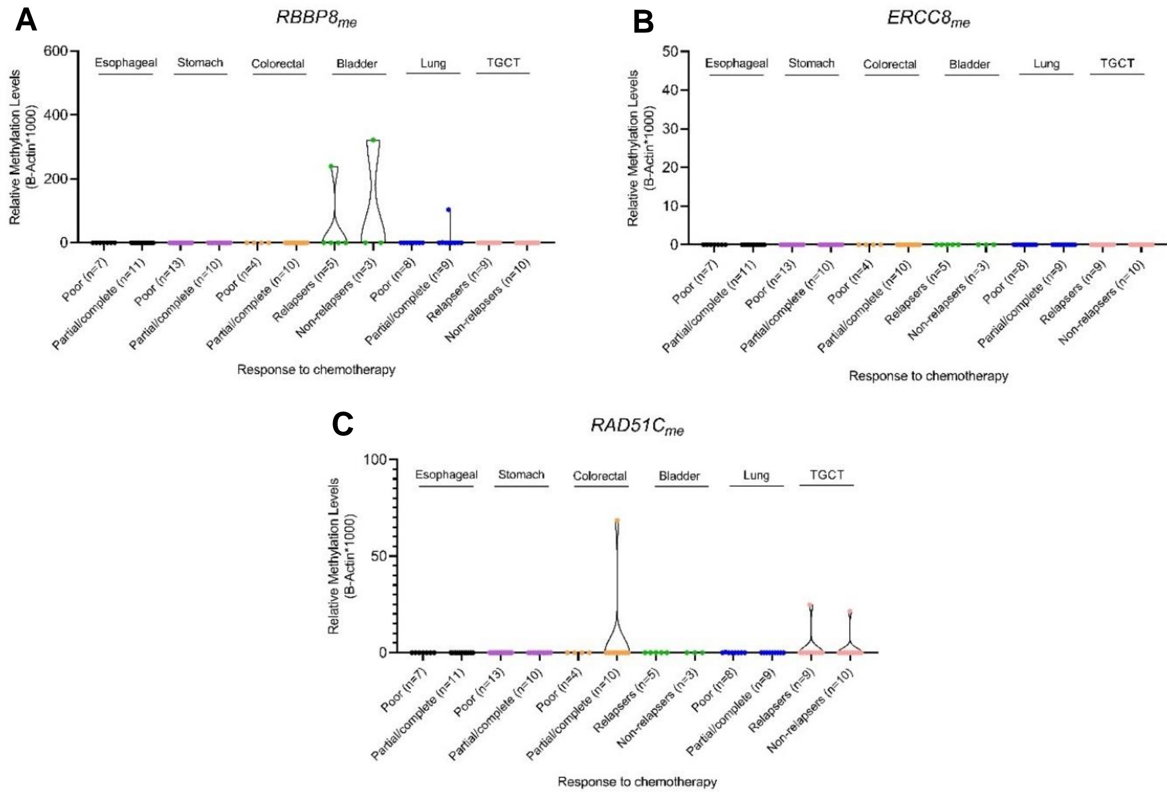
R=-0.218
LUSC-RBBP8-cg21074855
P=0.0000002
R=-0.329
LUSC-RBBP8-cg06299186
P=0.0001
R=-0.268
LUSC-RBBP8-cg16801093
P=0.0037
R=-0.205
LUSC-RBBP8-cg05305025
P=0.004
R=-0.204
LUSC-RBBP8-cg20891565
P=0.002
R=-0.216
Methylation (beta-values [450k])
mRNA expression (normalized_count)

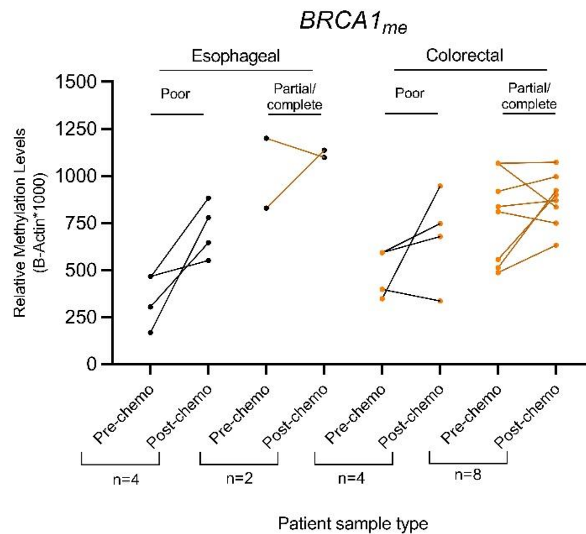


Supplementary Figure 1. Graphs of methylation vs gene expression for each selected CG site in esophageal cancer (A), stomach (B), colon and rectal (C), bladder (D), lung (E) cancers, and TGCT (F). Each point in the graph represents a

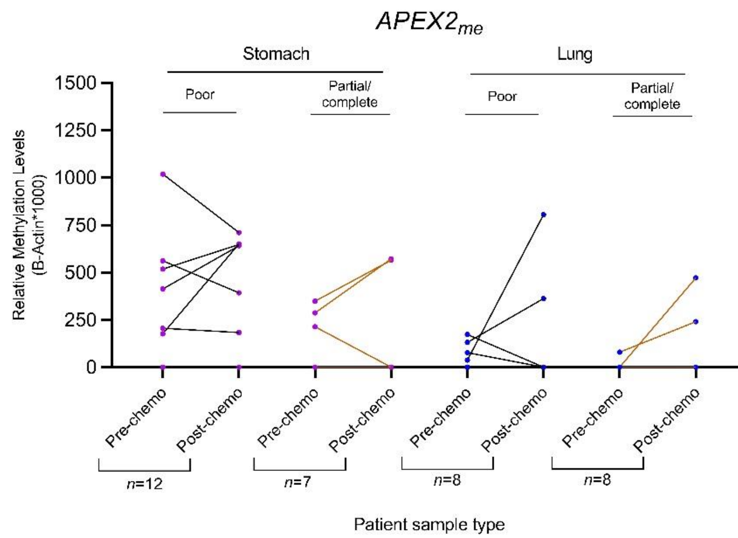
separate patient indicating the methylation levels of a specific CG. Blue line – locally weighted scatterplot smoothing, red line - median. CGs that are shared in several types of cancers are highlighted with colored frames, arrows indicate the CGs where qMSP primers and probes of DNA repair genes were localised (white arrows with black outline indicate CGs that were specific just to one cancer model and had qMSP primers and probes localised).



Supplementary Figure 2. Distribution of (A) *RBBP8* and (B) *ERCC8* and (C) *RAD51C* relative methylation levels in pre-chemotherapy samples of esophageal, stomach, colorectal, cladder, lung, and TGCT patients, with regards to chemotherapy response. Mann-Whitney U Test between poor and partial/complete (relapsers and non-relapsers in BC and TGCT). Red horizontal lines represent median methylation levels, n indicates the number of patient samples.



Supplementary Figure 3. Distribution of *BRCA1* relative methylation levels in pre- and post-chemotherapy samples (paired) of esophageal and colorectal cancer patients, regarding platinum chemotherapy response. Wilcoxon signed-rank Test between pre- and post-chemo. Red horizontal lines represent median methylation levels, n indicates the number of patients.



Supplementary Figure 4. Distribution of *APEX2* relative methylation levels in pre- and post-chemotherapy samples (paired) of stomach and lung cancer patients, regarding platinum chemotherapy response. Wilcoxon signed-rank Test between pre- and post-chemo. Red horizontal lines represent median methylation levels, n indicates the number of patients.