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"Pol theta: Study of resistance pathways in tumors associated with Homologous Recombination Deficiency (HRD)"

Doctoral Dissertation of:
Silvia Contino

Tutor:
Prof.ssa Lorena Incorvaia

The Chair of Doctoral Program:
Prof. Antonio Russo

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Abstract

Resistance to chemotherapy and PARP inhibitors remains a major challenge in the treatment of ovarian cancer. The introduction of PARP inhibitors has significantly improved the prognosis of patients with ovarian cancer, including those harboring BRCA1/2 mutations as well as Homologous Recombination Deficiency-positive and HRD-negative tumors. However, important questions remain regarding the adaptive pathways that enable tumor cells to develop resistance mechanisms. This study aimed to identify genes potentially involved in alternative DNA repair pathways that may cooperate with or compensate for HRR. We primarily focused on POLQ due to its central role in Theta-Mediated End Joining (TMEJ). Additionally, emerging evidence have drawn our attention to other genes, including APEX2, ALDH1A1, FANCD2, and FEN1. Our analyses evaluated patterns of deregulation and loss of expression of these genes across 2 patient cohorts (mutated cohort and wild-type cohort). Differential expression between HRD and HR-proficient tumors was observed for each gene, suggesting distinct adaptive repair strategies. These findings provide preliminary insight into the potential prognostic and predictive relevance of the analyzed genes and support further investigation into their role in drug resistance.

Summary

DNA repair systems play a crucial role in maintaining the integrity and stability of the double helix. When a DNA double-strand break (DSB) occurs, several repair mechanisms are activated to restore genomic integrity. These mechanisms are Non-homologous End Joining (NHEJ), Homologous Recombination Repair (HRR), and Microhomology-mediated End Joining (MMEJ) [3] [19] [21]. MMEJ includes highly specialized forms such as Theta-mediated End Joining (TMEJ) [28].

HRR is the highest-fidelity DSB repair pathway and plays a crucial role in cell survival [3]. Multiple genes are involved in the HRR pathway, and when one of these genes harbors a pathogenic variant (PV), dysfunction of the system may occur, resulting in the phenotype known as Homologous Recombination Deficiency (HRD) [5-6]. The HRD condition implies that other systems involved in DNA repair become upregulated and essential for cell survival. Furthermore, under HRD conditions, DNA single-strand break (SSB) repair systems assume a crucial role in cell survival.

PARP1, PARP2, and PARP3 enzymes play a critical role in the repair of SSBs through the base excision repair (BER) pathway and have become molecular targets of drugs that selectively inhibit their activity, thereby inducing synthetic lethality in HRD tumors [42]. To date, three PARP inhibitors (Olaparib, Niraparib and Rucaparib) have been approved for the treatment of ovarian cancer [45-57] [12]. However, despite encouraging results, the use of PARP inhibitors is also associated with the development of resistance. Molecular mechanisms such as reversion mutations, epigenetic modifications, restoration of ADP-ribosylation (PARylation), and pharmacological alterations that contribute to resistance are not yet fully understood [58]. Accordingly, the need to further investigate PARP inhibitor resistance and identify suitable prognostic and predictive biomarkers, as well as potential therapeutic targets, has emerged. Against this background, our study aimed to investigate the expression of

POLQ, ALDH1A1, FANCD2, FEN1, and APEX2 as genes cooperating in theta- mediated end joining (TMEJ) in ovarian cancer patients harboring BRCA1/2 or other HRR gene pathogenic variants, as well as in HR-proficient tumors. The rationale for choosing these genes stems from the evidence present in the literature. Tumors with alterations in HR genes exhibit elevated POLQ levels, and depletion of POLQ appears to be synthetically lethal in the context of HRD [18]. Consequently, Polθ has recently emerged as a promising therapeutic target in HRD tumors [78-79]. APEX2 has been described to contribute to MMEJ activity and has also been identified as a synthetic lethality-associated gene when linked to BRCA1/2 loss [27]. Studies have shown that increased expression of ALDH1A1 contributes to PARP inhibitor resistance in BRCA2-mutated ovarian cancer cells by enhancing MMEJ [75]. Furthermore, ALDH1A1 increases Polθ expression in ovarian cancer cells. ALDH1A1 catalyzes retinoic acid biosynthesis and promotes POLQ expression through activation of the retinoic acid signaling pathway, thereby maintaining MMEJ and contributing to PARP inhibitor resistance in HR-deficient ovarian cancer cells [76]. Kais et al. demonstrated that BRCA1/2-deficient tumors exhibit increased FANCD2 activity and that loss of FANCD2 enhances cell death, revealing a synthetic lethal relationship between FANCD2 and BRCA1/2 and highlighting its important role in replication fork protection [77]. Another study further clarified the role of DNA repair pathways in BRCA2-deficient tumor cells, showing that chemical inhibition of FEN1 selectively targets BRCA-deficient cells. FEN1 participates in MMEJ, supporting the concept of a close correlation between tumor cell survival and compensatory repair systems in HRD tumors [87-88]. Our analysis performed a baseline assessment of the expression of these genes, pre-treatment, and some of them showed dysregulation or absence of expression. The comparison between the two cohorts allowed us to hypothesize the different meanings that this deregulation may have in different contexts. This study may provide a foundation for future investigations into these genes as potential prognostic and predictive biomarkers.

CHAPTER 1

Background Rationale and Objectives

1.1 Genomic Instability and DNA Repair Pathways in Ovarian Cancer

1.1a Homologous Recombination Deficiency (HRD) in Ovarian Cancer: Prevalence, Genetic Drivers and Clinical Assessment.

High-grade serous ovarian cancer (HGSOC) is characterized by extensive genomic instability, which is primarily caused by alterations in genes encoding components of the DNA repair system [1]. Among the various repair mechanisms, the Homologous Recombination Repair (HRR) System appears to be the most frequently impaired in HGSOC, as approximately 50% of these tumors exhibit Homologous Recombination Deficiency (HRD) [2]. HRR is a highly conserved and crucial system for maintaining genomic stability. It uses a homologous DNA sequence—typically from the sister chromatid—as a template to repair double-strand breaks (DSBs) (Figure 1 A). This process is predominantly active during the S and G2 phases of the cell cycle, when sister chromatids are readily available [3]. The proper functioning of HRR system relies on the coordinated activity of several genes; pathogenic mutations or epigenetic alterations affecting these genes can lead to a state of HRD [2]. HRD represents a cellular phenotype determined by inefficient DSB repair and subsequent genomic instability [4]. HRD most commonly arises from mutations affecting BRCA1 and BRCA2, of which 15% are germline mutations and 6% are somatic mutations [5]. In addition, HRD can result from epigenetic mechanisms such as DNA methylation; promoter methylation of the BRCA1 gene has been reported in 5–30% of cases [6]. Alterations in other HRR genes—such as BRCA1, BRCA2, ATM, BARD1, BRIP1, CHEK1, CHEK2, FAM175A, MRE11A, NBN, PALB2, RAD51C, and RAD51D — can also lead to defective DNA repair and genomic instability. These non-BRCA HRR mutations are less frequent, but they may confer a similar HRD phenotype and sensitivity to PARP inhibitors [7] [8].

The hallmark of HRD-induced genomic instability is the presence of characteristic genomic scars (Figure 1 B), including loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large- scale state transitions (LST) [9] [10]. Detecting these genomic scars is crucial for treatment decisions, and this is possible thanks to several tests currently used in clinical practice. The goal is to predict the benefit from PARPi therapy even in the absence of detectable BRCA mutations. Current ESMO guidelines recommend testing for somatic BRCA1 and BRCA2 mutations as part of the HRD assessment, but this analysis alone is not sufficient [11]. In fact, the use of PARPi has also been approved in HRD-positive patients without BRCA1/2 mutations [12]. The PAOLA-1 trial validated the use of the myChoice® CDx assay (Myriad Genetics), making it the first FDA-approved platform. This assay analyzes genome-wide single nucleotide polymorphisms (SNPs). The resulting analysis of three parameters—LOH, TAI, and LST—is combined into a single HRD score. This score must typically exceed the predefined cutoff value of ≥ 42 to classify the tumor as HRD-positive [13] [14]. The SOLO-1 trial supported the use of the FoundationOne® CDx (Foundation Medicine) platform for BRCA mutation detection. F1CDx is based on next-generation sequencing (NGS)-based comprehensive genomic profiling (CGP) technology to examine 324 cancer genes, and it reports genomic loss of heterozygosity (gLOH) in ovarian cancer, using a predefined cutoff of 16% to classify tumors as HRD-positive [15]. Another possibility is represented by the SeqOne assay platform, which evaluates somatic CNVs across the genome and calculates an HRD score based on large-scale genomic instability patterns [16]. Although these tests are widely used and have revolutionized clinical practice in identifying patients who may benefit from PARP inhibitors, a major question remains. Using these assays does not allow us to identify cases in which DNA repair capacity has been regained through reversion mutations or epigenetic reactivation, despite a positive result. A positive HRD test merely indicates the presence of genomic scars, which reflect past events and do not provide real-time information on DNA repair functionality [17]. Furthermore, we must also consider the conditions in which non-HR repair pathways become activated in HRD-positive tumors and override HRR function, thereby inducing resistance [18].

1.1b Overview of Non-HR Repair Pathways (NHEJ, MMEJ/TMEJ)

HRR is not the only pathway responsible for repairing double-strand breaks (DSBs). Other pathways include the Non-homologous End-loining (NHEJ) system and the Microhomology-mediated End- Joining (MMEJ) system. NHEJ activity begins with the Ku70–Ku80 heterodimer, which is the first factor to bind to DSBs. The main difference between NHEJ and HRR is that NHEJ is active throughout the entire cell cycle, including non-replicative phases. NHEJ does not require a homologous template and therefore represents the default DSB repair pathway in both replicating and non-replicating cells. In contrast, HRR requires a homologous template and is active exclusively during the S and G2 phases of the cell cycle [19] [20]. MMEJ, also called the alternative non- homologous end-joining (Alt-NHEJ) pathway, has a distinctive property: it uses microhomologies sequences during end-joining. The microhomology sequences are short regions of complementary bases ranging from 2 to 20 nucleotides, they are exposed by end resection near the DSB. This activity represents the first step of the repair performed by MMEJ [21]. The MRN complex (MRE11, RAD50, and NBS1) performs this initial end-resection, as in HR [22]. The next step is represented by the coordinated activity of PARP-1, the MRN complex, and POLQ which performs end bridging and alignment of microhomology sequences [23]. This event generates non-homologous 3' tails. The activity of an endonuclease is essential for the removal of these tails and completion of the repair process. From the literature, it was initially hypothesized that the endonuclease responsible for this activity could be ERCC1/XPF (Excision Repair Cross-Complementing Group 1 / Xeroderma Pigmentosum Group F), as it participates in certain processes such as single-strand annealing [24]. However, new studies demonstrate that ERCC1 −/− cells do not exhibit a complete MMEJ defect. It is therefore possible that another endonuclease, namely flap endonuclease 1 (FEN1), is involved in the process. FEN1's activity is not limited to this. Indeed, it appears to play a crucial role as an endonuclease in the removal of displaced 5' ssDNA flaps created by PolQ activity [23-25]. PolQ acts during gap filling through its polymerase and helicase action, performing strand displacement followed by DNA synthesis [26]. Finally, as previously mentioned, FEN1 recognizes and cleaves the 5' ssDNA flaps produced. New evidence suggests that FEN1 may be a crucial protein for the proper functioning of MMEJ. In particular, BRCA2-deficient cells with upregulation of MMEJ have shown in vitro that FEN1 inhibition exhibits synthetic lethality. This suggests the potential use of this target in the treatment of BRCA-deficient tumors, either sequentially or in combination with PARP inhibitors [27]. The final step in the process is carried out by a ligase that seals the

DNA ends. Although several ligases have been identified at the cellular level, it appears that the one most strongly implicated in MMEJ is ligase III [23].

1.1c Focus on Polymerase Theta (Pol θ): Structure, Function, and Role in TMEJ

The central element of the MMEJ system is the activity of DNA polymerase theta (Polθ), hence the term theta-mediated end joining (TMEJ) [28] (Figure 2 [89]). Polθ is a 290-kDa family A DNA polymerase encoded by the POLQ gene. It has a unique structure, as it is the only eukaryotic DNA polymerase containing a helicase domain belonging to the superfamily 2 helicase family, known as Polθ-Hel. Polθ consists of an N-terminal helicase domain, a long unstructured central region, and a C-terminal polymerase domain. Each domain performs distinct functions that are essential for the proper execution of the MMEJ pathway. The N-terminal helicase domain (HD) performs DNA unwinding and annealing microhomologies sequences, scans and identifies short microhomologies, aligns microhomology regions, and stabilizes DNA end-bridging [29]. The unstructured central region mediates protein–protein interactions and thereby coordinates the assembly of the MMEJ repair complex [30]. Finally, the C-terminal polymerase domain (PD) carries out gap filling, strand- displacement DNA synthesis following microhomology alignment, and the generating 5' flap structures. It plays an essential catalytic role in completing MMEJ/TMEJ repair [31]. Polymerase theta activity, and the MMEJ double-strand break repair pathway in general, are intrinsically mutagenic [32]. Polymerase theta's mutational signature is represented by microhomology footprints and templated inserts. Microhomology footprints are short microhomologies sequences, typically 2–6 bp in length, that mark the junction between the two repaired DNA strands [33]. Templated inserts result from aborted template-dependent extension followed by re-annealing at secondary homologous sequences. The footprints reflect the mechanism of DNA end alignment, whereas templated inserts arise from aberrant and discontinuous DNA synthesis catalyzed by the polymerase [34]. This specific pattern of short insertions and deletions flanked by microhomologies corresponds to the small Insertion–Deletion Signature 6 (ID6), a statistical signature inferred from population-scale sequencing, which has been associated with TMEJ-dependent repair in cancer genomes analyzed by mutational signature frameworks, such as COSMIC. Evidence shows that tumors with high POLQ expression and

defective HR pathways exhibit an increased representation of this ID6 signature, reflecting the contribution of Polθ-mediated end joining to the mutational landscape [35]. Probably due to its mutagenic nature, POLQ expression in normal tissues is highly limited. Under physiological conditions, cells primarily rely on high-fidelity DNA repair pathways, such as HRR [18]. Conversely, under pathological conditions, including cancer, overexpression of Polθ has been observed [36]. In particular, Polθ overexpression has been documented in breast, ovarian, lung, bladder, colorectal, gastric, pancreatic, prostate, melanoma, uterine cancers, and glioblastomas, where it correlates with poor prognosis [37-39]. In breast and ovarian cancer, Polθ expression levels are particularly high in tumors exhibiting HRD. In the absence of functional HRR, cancer cells adapt by relying on alternative DNA repair pathways, such as TMEJ [40]. Polθ overexpression promotes tumor cell survival and proliferation and represents an unfavourable prognostic factor associated with a more aggressive tumor phenotype and an increased likelihood of disease recurrence [41]. Considering these characteristics, Polθ represents an attractive therapeutic target.

1.2 Therapeutic Targeting of HRD: PARP Inhibitors and Current Challenges

1.2a Synthetic Lethality and Mechanism of Action of PARP Inhibitors

The development of PARP inhibitors (PARPi) has represented a breakthrough in precision oncology. PARPi are NAD+ analogs that competitively inhibit the catalytic activity of PARP enzymes, thereby blocking the formation of poly (ADP-ribose) chains and preventing the recruitment of DNA repair proteins. In addition, PARPi trap PARP–DNA complexes, thereby interfering with replication fork progression [42]. Their mechanism of action is based on the inhibition of single-strand break (SSB) repair, leading to the accumulation of lethal double-strand breaks (DSBs) in HRD tumors. The development of PARPi stems from evidence demonstrating that the concomitant loss of PARP1 activity and BRCA1/2-mediated HR results in the progressive accumulation of DNA damage, ultimately leading to selective tumor cell death. This concept underlies the principle of synthetic lethality (SL) [43]. In cells with HRD, such as those harboring BRCA1 or BRCA2 mutations, in which accurate DSB repair is compromised, PARP inhibition acquires a central role in DNA repair and in the maintenance of

genomic stability. PARP is involved in the repair of DNA single-strand breaks; when PARP activity is inhibited, an accumulation of unrepaired single-strand DNA lesions occurs. The persistence of ssDNA breaks over successive cell cycles leads to cell cycle arrest at the G2/M checkpoint, ultimately resulting in cell death [43]. Another manifestation of synthetic lethality induced by PARP inhibition can be explained by the formation of unprotected ssDNA gaps, which can evolve into double-strand DNA breaks that cannot be efficiently repaired when BRCA genes are nonfunctional. These gaps arise because one of the physiological functions of PARP1 is to resolve unligated Okazaki fragments during lagging-strand DNA synthesis [44].

1.2b Clinical Application and Efficacy of PARP Inhibitors in Ovarian Cancer Treatment

Ovarian cancer was the first in which the FDA approved the use of a PARP inhibitor (PARPi). In 2014, phase II Studies 42 and 39 led to the FDA's accelerated approval of Olaparib in patients with BRCA1/2-mutated ovarian cancer with active disease who had received three or more prior lines of chemotherapy. These studies reported an overall response rate (ORR) of approximately 26–31% in patients with relapsed BRCA1/2-mutated ovarian cancer [45-46]. Subsequently, in 2017, the phase III SOLO-2 trial demonstrated that Olaparib significantly prolonged progression-free survival (PFS) in patients with relapsed high-grade serous ovarian cancer harboring BRCA1/2 mutations. This led to the approval of Olaparib as maintenance therapy in platinum-sensitive recurrent ovarian cancer, initially prioritized for patients with germline BRCA mutations [47]. The results of SOLO-2 were supported by additional trials, including SOLO-3 and OlympiAD, conducted in germline BRCA- mutated ovarian and breast cancers, respectively [48]. However, although the SOLO-3 study met its primary endpoint of ORR, no significant benefit was observed in terms of overall survival (OS). This finding contributed to the FDA's decision in 2022 to withdraw the indication for Olaparib as active treatment in relapsed ovarian cancer [49]. The approved use of Olaparib remains in the maintenance setting, including first-line maintenance in newly diagnosed advanced ovarian cancer with BRCA mutations, as demonstrated by the SOLO-1 study [50]. Furthermore, the PAOLA-1 trial expanded the population eligible for Olaparib by supporting its approval, in combination with Bevacizumab, in patients with homologous recombination deficiency (HRD)–positive tumors beyond BRCA1/2 mutations [12]. In 2017, the NOVA trial demonstrated a

progression-free survival (PFS) benefit, supporting the use of Niraparib as maintenance therapy in platinum-sensitive recurrent ovarian cancer, independent of BRCA mutation status [51]. Subsequently, in 2020, the FDA approved Niraparib as first-line maintenance therapy in advanced ovarian cancer based on the results of the phase III PRIMA study. In PRIMA, a PFS benefit was observed in patients with BRCA mutations and in those with homologous recombination deficiency (HRD), and to a lesser extent in the overall population [52]. Rucaparib is another PARP inhibitor whose indication in the recurrent treatment setting was withdrawn following results from the ARIEL4 study, which showed no overall survival (OS) benefit compared with standard chemotherapy [53]. In 2018 received accelerated approval as monotherapy for the treatment of recurrent ovarian cancer, based on the phase II ARIEL2 study. This approval followed the earlier phase III ARIEL3 trial, which had established the efficacy of Rucaparib as maintenance therapy after response to platinum-based chemotherapy [54-56]. More recently, the ATHENA-MONO study provided evidence supporting the use of Rucaparib in the first-line maintenance setting, particularly in patients with HRD-positive disease, thanks to these results, Rucaparib was approved as first-line maintenance therapy in 2025 [57]. Taken together, these studies highlight the critical importance of distinguishing the disease setting in which PARP inhibitors are used, particularly between first-line maintenance and recurrent disease settings.

1.2c Mechanisms of Acquired and Intrinsic Resistance in HRD Tumors

Although the use of PARP inhibitors in the treatment of ovarian cancer is now well established, their effectiveness is limited by clinical challenges related to the development of resistance mechanisms. When discussing PARP inhibitor resistance, a distinction must be made between primary and acquired resistance. Primary resistance refers to the lack of an initial clinical response to PARP inhibitor treatment, observed from the onset of therapy. In this context, despite the presence of biomarkers theoretically predictive of sensitivity, the tumor does not derive benefit from PARP inhibition, and disease progression occurs early. Conversely, acquired resistance develops after an initial response to PARP inhibitor treatment. In this case, the tumor is initially sensitive, but sustained therapy exerts selective pressure that promotes the emergence of resistant clones, ultimately leading to disease recurrence or progression [58]. Primary resistance to PARP inhibitors may arise from pre-

existing biological features, including a discrepancy between genomic HRD signatures and functional HRR activity. Although HRD status can be detected using currently available diagnostic techniques, this does not provide absolute certainty that HRR system is functionally impaired at the time of treatment. Indeed, the accumulation of genomic scars reflects historical defects in HR rather than real-time repair capacity and may therefore result in misclassification of tumors as HRD-positive despite preserved HR functionality [59]. In addition, the literature describes hypomorphic BRCA1/2 mutations that retain partial DNA repair activity, highlighting that not all BRCA mutations are functionally equivalent and that some variants may preserve residual HRR function [60]. Typical mechanisms of acquired resistance include events that lead to restoration of HRR system function. The most frequently reported mechanism is the acquisition of reversion mutations in BRCA1/2, resulting in the re-expression of functional BRCA1/2 proteins and reactivation of HR pathways [61]. Reversion mutations may also affect other genes of the HRR system, although less frequently [62]. These mutations include second-site insertions or deletions, as well as in-frame deletions that restore the original reading frame. In addition, evidence suggests that within each gene there may be regions more prone to reversion events, representing mutational “hotspots” where such alterations are more likely to occur [63]. According to the literature, reversion mutations have been detected in approximately 20–40% of cases of acquired PARP inhibitor resistance, while in less than 5% in platinum-sensitive settings [64-65]. In this context, liquid biopsy has emerged as a promising and innovative tool for the early detection of reversion mutations, potentially preceding radiological or clinical disease progression [64] [66]. Epigenetic events also contribute to the HRD phenotype and may be subject to dynamic modification during PARP inhibitor treatment. Several studies have shown that loss of BRCA1 promoter hypermethylation is associated with PARP inhibitor resistance [67]. Additional mechanisms leading to BRCA1/2-independent restoration of HR have also been described. Among these, restoration of replication fork protection represents a key process; both BRCA1 and BRCA2 play essential roles in protecting stalled or reversed replication forks from nucleolytic degradation. It has been proposed that activation of the ATR–CHK1 signalling pathway promotes cell cycle arrest and stabilization of stalled replication forks, thereby contributing to resistance independently of HR restoration [68]. Another resistance mechanism involves modulation of PARP1 expression or function; downregulation of PARP1 or alterations in its DNA- binding domains reduce PARP trapping and consequently diminish the cytotoxic

efficacy of PARP inhibitors. Finally, there is evidence that overexpression of ABCB1, leading to increased transcription of the drug efflux pump MDR1 (P-glycoprotein), results in enhanced drug efflux and reduced intracellular PARP inhibitor concentrations, thereby promoting resistance [69]. In the next paragraph, we will focus on the central topic of this thesis, namely the upregulation of alternative DNA repair pathways—particularly Polθ-mediated end joining (TMEJ)—as a major contributor to resistance to PARP inhibitors. Activation of alternative repair pathways, including TMEJ/MMEJ, can contribute to both primary and acquired resistance; the distinction depends on the temporal and biological context in which the alternative repair system predominates. Notably, a subset of HRD tumors exhibits high basal expression of POLQ and therefore a strict dependence on pre-existing MMEJ/TMEJ in the absence of functional HRR. In these cases, the tumor is not truly dependent on HR, and as a consequence, the synthetic lethality induced by PARP inhibitors is ineffective. Conversely, in the majority of cases, TMEJ is selected or further enhanced during PARP inhibitor treatment. PARP inhibitors induce replication stress with consequent accumulation of double-strand breaks (DSBs), thereby allowing the emergence of tumor cell clones with adaptive upregulation of POLQ under selective pressure. As a result, TMEJ becomes the dominant DNA repair pathway [18] [32] [70].

1.3 Alternative DNA Repair Pathways Driving Resistance to PARP Inhibitors

1.3a Polymerase Theta-Mediated End Joining (TMEJ) as the Primary Compensatory Survival Pathway: Why Target Polθ to Trigger Synthetic Lethality?

Synthetic lethality underlies the mechanism of action of PARP inhibitors. When this interaction is lost, therapeutic resistance develops. Restoring or re-establishing synthetic lethality therefore represents a potential strategy to overcome resistance to PARP inhibitor treatment. In this context, increasing attention is being directed toward alternative compensatory DNA repair mechanisms that are activated when BRCA1/2 or other components of the HRR pathway are deficient. It is well established that tumor cells can exploit alternative DNA repair pathways, such as polymerase theta– mediated end joining (TMEJ), as compensatory

mechanisms. These mechanisms allow tumor cells to evade synthetic lethality; however, the resulting hyper-dependence on these pathways may itself represent a therapeutic vulnerability for the tumor [70]. Evidence indicates the existence of synthetic lethal interactions between POLQ and multiple DNA repair genes, including key factors involved in homologous recombination, such as BRCA1 and BRCA2 [18] [71] [27]. Although preclinical studies have shown that inhibition of POLQ is associated with impaired cell viability, suggesting the activation of a potential synthetic lethality mechanism, the precise molecular processes underlying this effect remain poorly understood to date [18] [71-72]. The literature proposes two models to explain the synthetic lethality between POLQ and the HR pathway. In the first model, HR-deficient tumor cells are highly dependent on POLQ due to its function in TMEJ, which becomes the primary repair mechanism for double-strand breaks (DSBs). Because of continuous proliferation, tumor cells accumulate chronic replication stress, resulting in replication fork collapse and an increased burden of DSBs, which cannot be efficiently repaired by HRR. When key components of this pathway, such as LIG3 and LIG1, are inhibited, a synergistic effect with PARP inhibitors is observed, ultimately leading to cell death. In the second model, which can be defined as the RAD51 model, the dependence on POLQ derives not only from its role in TMEJ, but also from its activity as a RAD51 antagonist. Under normal conditions, RAD51 promotes DNA repair through HR. POLQ, in contrast, limits RAD51 activity, thereby directing repair pathway choice toward TMEJ. In this context, POLQ activity is essential to prevent the cell from being driven toward a non-functional repair pathway [73]. A particularly interesting development is the evidence showing that loss of POLQ can be detrimental even in cells with a functional HR pathway, suggesting that HR is not always sufficient to compensate for the absence of TMEJ. This observation broadens the therapeutic landscape for the potential use of POLQ inhibitors [40]. Currently, efforts are underway to develop POLQ inhibitors; however, the optimal therapeutic target—whether the helicase domain or the polymerase domain—has not yet been clearly defined [73]. Recent evidence from the literature indicates that sensitivity to Polθ inhibitors depends not only on the gene conferring HRD, but also on the type of mutation involved. In particular, BRCA1 mutant alleles proficient in DNA end resection have been shown to be significantly more dependent on Polθ for cell viability. This observation opens important avenues for further research into how the type and genomic location of mutations in HRR genes influence sensitivity to targeted therapeutic strategies [78].

1.3b Emerging Resistance-Associated Genes: ALDH1A1, FEN1, APEX2, FANCD2

Polθ has also been shown to engage in synthetic lethal interactions with other DNA repair–related genes involved in DSB repair and HR. One study identified the nuclease APEX2 as a functional effector of the MMEJ pathway. Specifically, APEX2 acts at multiple steps to support the survival of HR-deficient cells, thereby emerging as a particularly promising therapeutic target for the elimination of HRD cancer cells, either as an alternative to or in combination with Polθ or PARP inhibitors [74]. Liu L et al demonstrated that increased expression of ALDH1A1 contributes to PARPi resistance in BRCA2-mutated ovarian cancer cells by enhancing MMEJ [75]. In a subsequent study, the same authors observed that ALDH1A1 increases Polθ expression in ovarian cancer cells. Specifically, ALDH1A1 catalyzes Retinoic Acid (RA) biosynthesis and promotes POLQ expression through activation of the RA signaling pathway, thereby sustaining MMEJ activity and contributing to PARP inhibitor resistance in HR-deficient ovarian cancer cells. Accordingly, combined treatment with olaparib and an ALDH1A1 inhibitor was shown to synergistically reduce tumor cell viability in BRCA1/2-mutated ovarian cancer cells with high ALDH1A1 expression [76]. Kais et al. demonstrated that BRCA1/2-deficient tumors exhibit increased FANCD2 activity. Loss of FANCD2 in BRCA1/2-deficient tumor cells results in increased cell death. This study reveals a synthetic lethal interaction between FANCD2 and BRCA1/2 and highlights an important role for FANCD2 in replication fork protection and stability [77]. A study has further elucidated the role of alternative DNA repair pathways in BRCA2-deficient tumor cells, showing that chemical inhibition of FEN1 selectively targets BRCA-deficient cells. FEN1 is an essential component of the MMEJ pathway, and its inhibition compromises the survival of HR-deficient cells. These findings further support the concept that tumor cell survival in the context of homologous recombination deficiency (HRD) is critically dependent on collateral DNA repair systems [27]. Recent evidence from the literature indicates that sensitivity to Polθ inhibitors depends not only on the gene conferring HRD, but also on the type of mutation involved. In particular, BRCA1 mutant alleles proficient in DNA end resection have been shown to be significantly more dependent on Polθ for cell viability. This observation opens important avenues for further research into how the type and

genomic location of mutations in HRR genes influence sensitivity to targeted therapeutic strategies [78].

1.4 Objectives

Based on the aforementioned data, the aim of this project is to investigate the role and expression of POLQ, ALDH1A1, FANCD2, FEN1, and APEX2 as genes cooperating in theta-mediated end joining (TMEJ) in ovarian cancer patients harboring BRCA1/2 or other HRR gene pathogenic variants, as well as in HR-proficient tumors. Furthermore, we sought to investigate the potential prognostic role of the expression levels of these genes on clinical outcomes, based on the results obtained.

CHAPTER 2
Patients and Methods

2.1 Population study

In our study, conducted at the Department of Medical Oncology of the "Policlinico Paolo Giaccone" in Palermo, we collected and analyzed clinical, laboratory, and genetic data from 72 patients with ovarian cancer tested for BRCA1/2 or other HRR genes, between January 2020 and March 2025, as part of routine clinical practice. Eligibility for genetic counseling and testing was determined in accordance with international and national guidelines and clinically available risk assessment tools, taking into account personal and family cancer history, including age at diagnosis, presence of multiple primary tumors, number of affected relatives, and tumor molecular characteristics.

The variables of interest included in the database were as follows:

i. Clinical data: age at diagnosis; surgical treatment; tumor bilaterality; histological subtype; tumor grade; FIGO and TNM stage; personal history of other malignancies; family history; and chemotherapy treatments received.
ii. Genetic data: tissue used for testing; presence of tumor pathogenic or likely pathogenic variants; variant allele frequency; germline BRCA pathogenic variants; germline pathogenic variants in non-BRCA HRR genes; mutation nomenclature (HGVS); protein change; variant classification and HRD Myriad score.

2.2 Mutational analysis

From the routine tissue used for BRCA/HRR gene analysis, reverse transcription and gene expression analysis were performed using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad), following the manufacturer's protocol. Positive and negative droplets were counted, and QuantaSoftTM software calculated the concentration of target RNA as copies/µL. Only reactions with a total number of events (corresponding to the number of droplets generated)

greater than 10,000 were considered. The relative expression value of each gene was calculated as the ratio of droplet counts for the gene of interest to those of the reference gene. The optimized enzyme blend enabled partitioning of RNA samples into droplets while keeping the enzymes inactive until the reverse transcription reaction was performed at 50 °C. This approach enhanced specificity and efficiency by ensuring full enzyme activation during primer-mediated cDNA synthesis. The supermix contained an RNase inhibitor that protected the RNA throughout the entire workflow. GAPDH was used as the housekeeping reference gene. Hydrolysis assays included a fluorescently labeled, sequence-specific oligonucleotide probe (TaqMan). After reverse transcription, the resulting cDNA was amplified for target detection using TaqMan hydrolysis probes. After PCR amplification, each droplet generated a fluorescent positive or negative signal, indicating whether the target gene was present or absent after partitioning. Each droplet provided an independent digital measurement.

2.3 Statistical analysis

The expression levels of POLQ, APEX2, ALDH1A1, FANCD2 and FEN1 genes were analyzed by stratifying the patients enrolled in this study according to their molecular characterization, specifically the presence or absence of pathogenic BRCA1/2 variants, prior to the initiation of treatment. A paired Wilcoxon test was used to compare the median expression levels of the five genes between BRCA1/2- mutant patients and those without pathogenic genetic alterations. All statistical analyses were performed using RStudio software, Version 2023.03.0+386 (2023.03.0+386), Copyright (C) 2022 by Posit Software, PBC.

CHAPTER 3

Results

3.1 Clinical characteristics of the patients

The collected data corresponded to 72 patients, who were divided into three cohorts: 21 patients carrying pathogenic or likely pathogenic BRCA1/2 variants [B1/2 cohort]; 10 patients in the HRD- positive cohort (BRCA1/2 wild type) [HRD cohort]; and 41 patients in the wild-type cohort [WT cohort] (Table 1 and Table 3). From this database, analyses were performed on suitable tissue samples, and only reactions with a total number of events (corresponding to the number of droplets generated) greater than 10,000 were included. This selection resulted in a final dataset comprising 6 patients with pathogenic or likely pathogenic BRCA1/2 variants [B1/2 cohort]; 3 patients in the HRD-positive cohort (BRCA1/2 wild type) [HRD cohort]; and 10 patients in the wild-type cohort [WT cohort]. The median age (65) at diagnosis was higher in the HRD cohort, in B1/2 cohort and WT cohort the median age was 58 and 56 respectively. In the B1/2 cohort, 5 patients had a high-grade serous histology and 1 case had a high-grade endometrioid histology; in the HRD cohort, all 3 patients had a high-grade serous histology; finally, in the WT cohort, the histologist were divided as follows: 3 serous low grade; 5 serous high grade; 2 endometrioid (Table 2). From a mutational perspective, the most frequent event was frameshift mutations, identified in 4 out of 6 samples: specifically, 2 in BRCA1 and 2 in BRCA2. Variant allele frequency levels were particularly high in all six samples analyzed (Table 4).

3.2 Expression of POLQ, APEX2, ALDH1A1, FANCD2, FEN1 in EOC

To discuss the results regarding the expression of the genes we assessed, we merged the two patient cohorts: B1/2 cohort and HRD cohort. We then compare the mutated cohort (B1/2

cohort + HRD cohort) with the WT cohort.

POLQ

POLQ expression was almost similar between the mutated cohort and the WT cohort (Table 5). There was 1 outlier in the WT cohort with a value of 22.52. In the mutated cohort, the gene was dysregulated in 4 out of 9 cases (44%), and in 5 cases it was null (56%). The median and the mean are respectively 0 [0 – 2,31] and 1,138. In the WT cohort, the gene was dysregulated in 4 out of 10 cases (40%), and in 6 cases it was null (60%). The median and the mean are respectively 0 [0 – 1,478] and 2,81. The median and mean of all the cohorts together are and respectively 0 [0 - 1,995] and 2,017 (P-value 0,8916) (Figure 3).

APEX2

APEX2 expression was higher in the WT cohort than in the mutated cohort (Table 6). There were 2 outliers in the mutated cohort with a value of 12,4 and 14,37; and 1 outlier in the WT cohort with a value of 33,43. In the mutated cohort, the gene was dysregulated in 6 out of 9 cases (67%), and in 3 cases it was null (33%). The median and the mean are respectively 2,86 [0 – 3,88] and 4,19. In the WT cohort, the gene was dysregulated in 8 out of 10 cases (80%), and in 2 cases it was null (20%). The median and the mean are respectively 8,13 [2,5 – 12,44] and 9,36. The median and mean of all the cohorts together are and respectively 3,30 [0,47 -11,55] and 6,914 (P-value 0.3032) (Figure 4).

ALDH1A1

ALDH1A1 expression was higher in the WT cohort than in the mutated cohort (Table 7). There was 1 outlier in the mutated cohort with a value of 734,44; and 1 outlier in the WT cohort with a value of 372. In the mutated cohort, the gene was dysregulated in 8 out of 9 cases (89%), and in 1 case it was null (11%). The median and the mean are respectively 5,23 [2,86 – 135] and 119,56. In the WT cohort, the gene was dysregulated in 8 out of 10 cases (80%), and in 2 cases it was null (20%). The median and the mean are respectively 11,91 [5,26 – 14,58] and 46,25. The median and mean of all the cohorts together are and respectively 9,98 [3,165 – 35,35] and 80,977 (P-value is 0.9024) (Figure 5).

FANCD2

FANC2 expression was significantly higher in the WT cohort than in the mutated cohort (Table 8). There were 2 outliers in the mutated cohort with a value of 27,53 and 78,85. In the mutated cohort, the gene was dysregulated in 3 out of 9 cases (33%), and in 6 cases it was null (67%). The median and the mean are respectively 0 [0 – 8,97] and 12,82. In the WT cohort, the gene

was dysregulated in 7 out of 10 cases (70%), and in 3 cases it was null (30%). The median and the mean are respectively 13,35 [3,2 – 46,49] and 28,27. The median and mean of all the cohorts together are and respectively 8,97 [0 – 34,76] and 20,95 (P-value 0.1309) (Figure 6).

FEN1

FEN1 expression was higher in the WT cohort than in the mutated cohort (Table 9). There was 1 outlier in the mutated cohort with a value of 59.17. In the mutated cohort, the gene was dysregulated in 6 out of 9 cases (67%), and in 3 cases it was null (33%). The median and the mean are respectively 11,68 [0 – 19,23] and 14,78. In the WT cohort, the gene was dysregulated in 7 out of 10 cases (70%), and in 3 cases it was null (30%). The median and the mean are respectively 18,1 [3,9 – 33,31] and 22,74. The median and mean of all the cohorts together are and respectively 16,79 [0 -26,60] and 18,97 (P-value 0.4069) (Figure 7).

Subgroup analysis

For the WT cohort three subgroups were identified considering histotype: Low Grade Serous; High Grade Serous; Endometrioid. The median and mean of POLQ expression were respectively 0.87 and 5.2 for Low Grade Serous; 0.938 for High Grade Serous; 0 for Endometrioid (Table 10). The median and mean of APEX2 expression were respectively 9.23 and 8.04 for Low Grade Serous; 2,81 and 4,6 for High Grade Serous; 23,225 for Endometrioid (Table 11). The median and mean of ALDH1A1 expression were respectively 9.98 and 9.41 for Low Grade Serous; 13,83 and 12,45 for High Grade Serous; 186 for Endometrioid (Table 12). The median and mean of FANCD2 expression were respectively 0 and 27.33 for Low Grade Serous; 13,58 and 31,72 for High Grade Serous; 20.99 for Endometrioid (Table 13). The median and mean of FEN1 expression were respectively 19.37 and 17.81 for Low Grade Serous; 15,6 and 16,67 for High Grade Serous; 45,315 for Endometrioid (Table 14).

CHAPTER 4

Discussion

Based on the literature, we identified POLQ, ALDH1A1, FEN1, APEX2, and FANCD2 as genes of interest. We then assessed their baseline expression at the time of diagnosis in ovarian cancer patients. We explored the potential use of gene expression levels as prognostic and predictive markers for response to chemotherapy (platinum-based) and PARPi treatment. Furthermore, based on data reported in the literature, the selected genes exhibited characteristics that suggest they may represent potential therapeutic targets, either in combination with PARP inhibitors or during disease progression under PARPi treatment. In selecting these genes, we placed particular emphasis on POLQ, given the extensive evidence supporting its role and activity within theta-mediated end joining (TMEJ). The literature consistently reports that cancer cells often exhibit elevated POLQ expression, a feature that is more pronounced in HRD tumors [18]. In this context, HRD increases biological dependence on Polθ, resulting in POLQ upregulation and marked vulnerability to its inhibition through synthetic lethality mechanisms. An important note is that not all HRD tumors necessarily display high POLQ expression; rather, this feature is expected to become more evident as a consequence of selective pressure induced by chemotherapy and PARPi treatments [79]. In our baseline (pretreatment) analysis, POLQ it is observed to be deregulated in few cases, and its expression was overall homogeneous among mutated cohort (BRCA1/2-mutated + HRD-positive non-BRCA1/2) (median 0) and Wild-Type cohort (median 0). A current limitation in the literature is the lack of robust direct evidence comparing POLQ expression levels before and after chemotherapy or PARPi treatment, which would provide quantitative data on dynamic changes in expression. Based on available evidence, we can hypothesize that low POLQ expression may render tumors more susceptible to both chemotherapy and PARP inhibitors, particularly in an HRD setting. However, baseline POLQ assessment should not be considered absolute, given the tumor's capacity to activate adaptive and selective mechanisms in response to therapeutic pressure. In ovarian cancer (OC), evidence

specifically addressing low POLQ expression remains limited. A recently published study by Espín et al. demonstrated in vitro in HRD cancer cells that microenvironmental factors can modulate POLQ expression, showing in particular that hypoxia reduces POLQ levels. Consequently, the biological significance of what may be defined as a POLQ-low state varies depending on the cellular context [80]. In HR-deficient tumors, POLQ-low may reflect a condition of pronounced genomic fragility associated with impairment of key DNA repair pathways. When observed—as in our dataset—this state could correspond to a transient, subclonal event with functional relevance, potentially conferring increased sensitivity to treatment. Conversely, POLQ-low expression in HR-proficient tumors is more consistent with a phenotype characterized by lower chromosomal instability, reflecting preferential reliance on the high-fidelity, low-error homologous recombination repair system. Our findings suggest that baseline assessment of POLQ expression alone may be insufficient to predict therapeutic response. Instead, POLQ should be considered within the broader context of HR status and tumor adaptability, highlighting the need for dynamic or longitudinal evaluation to better capture DNA repair dependencies in ovarian cancer. We found a strong rationale in the literature for combining the analysis of four additional genes with the POLQ study: ALDH1A1, FEN1, APEX2, and FANCD2.

APEX2 is an endonuclease primarily involved in base excision repair (BER) and in the management of oxidative damage and replication stress. APEX2 has been reported to contribute to MMEJ activity, influencing DNA double-strand break (DSB) repair and thus potentially playing a role in tumor genomic stability [74]. In this regard, Mengwasser et al. (2019) identified APEX2 as a gene whose suppression induces synthetic lethality in EOC models harboring pathogenic BRCA1/2 variants [27]. In our study, we observed distinct APEX2 expression profiles, with a higher median expression in the wild-type cohort (median 8,13) compared to the mutated cohort (median 2,86). While the literature reports that APEX2 may display abnormal expression across multiple cancer types (including kidney, breast, lung, liver, and uterine tumors), our analysis specifically evaluates EOC, distinguishing between HR-proficient and HRD tumors [81]. In our wild type cohort APEX2 expression tended to be higher suggesting a potential association with this molecular background. Overall, APEX2 dysregulation may be informative to treatment response and disease behaviour. Based on its known role in DNA damage processing, lower APEX2 expression has been suggested to be associated with increased sensitivity to DNA-damaging agents in preclinical models.

Therefore, future studies comparing baseline and peri-treatment APEX2 expression may help clarify its dynamic regulation and clinical relevance.

ALDH1A1 contributes to the maintenance of cellular homeostasis under oxidative stress by promoting the activation of alternative DNA repair pathways. The literature supports this role in EOC, where increased ALDH1A1 activity has been associated with sustained cell viability and greater tumor aggressiveness. Baseline immunohistochemical evaluation in EOC has further shown that ALDH1A1 upregulation is associated with poor prognosis, with lower overall survival (OS) and progression-free survival (PFS) compared with tumors with lower expression [82-83]. In our analysis, ALDH1A1 dysregulation was observed in baseline, pre-treatment samples, with a higher median expression level in the wild-type cohort (median 11,91) compared with the mutated cohort (median 5,23). However, a discrepancy between median and mean values was noted between the two groups, as the mean expression was higher in the mutated cohort (mean 119,56) vs wild type cohort (mean 46,25). This divergence suggests increased heterogeneity within the mutated cohort, characterized by a subset of samples exhibiting markedly elevated expression levels, while the majority of cases displayed low expression. The presence of ALDH1A1 dysregulation supports its potential role as a prognostic marker. The higher median expression observed in the wild-type cohort suggests that ALDH1A1 assessment may provide additional prognostic information in HR-proficient tumors. Considering the unfavourable prognosis associated with high ALDH1A1 expression, and the increased sensitivity to chemotherapy reported in cases of gene downregulation, baseline assessment of ALDH1A1 may offer insights into treatment response [84-85]. ALDH1A1 dysregulation appears to reflect an intrinsic tumor characteristic that emerges early during tumor evolution, although it may also be further upregulated following treatment as an adaptive response. Preclinical studies by Liu et al. (2020) and Lavudi et al. (2023) demonstrated that ALDH1A1 inhibition exerts a synergistic effect when combined with PARP inhibitors [75-76]. Considering our baseline findings, these observations suggest that pre-treatment evaluation alone may be insufficient, and that post-treatment assessment of ALDH1A1 expression could be informative for its potential exploitation as a therapeutic target.

FANCD2 is a central component of the Fanconi Anemia pathway and is essential for replication fork protection and the management of replicative stress. Its function parallels that of HR; however, it does not restore HR activity, and its upregulation can instead be viewed as an adaptive dependency. Kais et al. (2016) demonstrated that loss of FANCD2 induces a

synthetic lethal interaction in tumors harboring BRCA1/2 loss [77]. The HR-proficient cohort presented the highest median FANCD2 expression (median 13,35), suggesting that FANCD2 may be selectively maintained as a cooperator of HR in these tumors. Surprisingly, we observed undetectable FANCD2 expression in more than half of the mutated cohort (median 0); furthermore, observing within the mutated cohort (BRCA1/2 mutated + HRD-positive no BRCA1/2) all three HRD-positive no BRCA1/2 samples showed null expression of FANCD2. The differential expression of FANCD2 observed between HRD and HR-proficient tumors may reflect distinct adaptive strategies to replication-associated DNA damage. While HR-proficient tumors may retain high FANCD2 expression to support fork stability and faithful DNA repair, the biological rationale for HR-deficient tumors exhibiting low or absent FANCD2 expression remains less clear. Our baseline assessment may capture a clonal population that is subsequently subjected to selective pressure during tumor evolution. Given its biological role, low expression or complete inactivation of FANCD2 in HRD tumors may reflect an impaired capacity to protect stalled replication forks, thereby exacerbating replication-associated DNA damage. The literature indicates that cellular models of HGSOC with FANCD2 upregulation display resistance to platinum-based chemotherapy, whereas models with low FANCD2 expression show increased sensitivity [86]. This condition may therefore enhance vulnerability to DNA-damaging agents and PARP inhibitors, representing a potential biomarker of therapeutic sensitivity. However, such a state may also be poorly tolerated by tumor cells, which could explain why complete loss of FANCD2 is rarely observed in advanced tumors and may instead represent a condition detectable predominantly at the baseline stage. FEN1 is an endonuclease essential for the maturation of Okazaki fragments and for base excision repair (BER). Specifically, after the MRN complex and POLQ perform end-bridging and alignment of microhomology sequences, non-homologous 3' tails are generated and subsequently removed by endonuclease activity primarily mediated by FEN1 [23] [25]. FEN1 is frequently overexpressed at baseline levels in tumors, regardless of HRD status, to sustain replication-associated stress. In HRD tumors, it becomes a crucial determinant of cell survival, given its central role in TMEJ function [87]. The literature indicates that its activity is critical in BRCA2-deficient cells with upregulation of MMEJ, where in vitro studies have demonstrated that FEN1 inhibition results in synthetic lethality [27]. In HRD cellular models, FEN1 inhibition causes accumulation of DNA damage, thereby reducing cell survival. Our analysis identified deregulated FEN1 expression in both cohorts, with higher expression levels in the wild-type

cohort (median 18,1) vs wild type cohort (median 11,68). Assessing FEN1 expression in the context of existing literature provides information regarding therapeutic prognosis and potential sensitivity to treatments [88]. Our data are consistent with current evidence and suggest that HR-proficient tumors may benefit from the incorporation of novel prognostic biomarkers. From the subgroup analysis we performed, it was observed that for APEX2; ALDH1A1; FANCD2 and FEN1 genes de-regulated differently in relation to histotype. The Endometrioid histotype showed a higher median than the others histotype (APEX2 median 23.22; ALDH1A1 median 186; FANCD2 median 20.99; FEN1 median 45.315). Although to date there is no evidence in the literature that allows us to interpret what has been described, but his difference could underlie gene modulation differences related to histotype. The relatively small sample size represents a limitation of the study and may partially explain the lack of statistical significance ($p > 0.05$). In conclusion, our study provides a baseline snapshot of gene expression patterns that are closely associated with alternative DNA repair systems activated by tumor cells in HRD conditions and in HR-proficient settings (Figure 8 A and B). This study opens several avenues for further investigation. First, comparison with healthy tissue could help define the extent to which such deregulation diverges from physiological conditions and clarify the underlying mechanisms. Second, post-treatment reassessment may provide valuable insights into tumor behaviours under drug-induced selective pressure. Future studies may validate and standardize these genes as potential prognostic and predictive biomarkers.

CHAPTER 5

Tables and Figures

Table 1 The PV/LPVs of BRCA1/2 genes in ovarian cancer patients.

Gene	Type of variant	Molecular consequence	HGVS Nomenclature	Protein change	VAF (%)	Variant Class (PV/LPVs)
BRCA1	SNV	NS	c.1154G>A	p.W385Ter	67.2	5
BRCA1	SNV	M	c.5297 es21	p.Ile1766Ser	92.02	5
BRCA1	Deletion	Fs	c.3736delA es11	p.Thr1246ProfsTer18	79.78	5
BRCA1	Deletion	Fs	c.4964_4982del 5083del19	p.Ser1655TyrfsTer16	-	5
BRCA1	Deletion	Fs	c.3822_3858del	p.Ile1275Argfs20	78	Unclassified
BRCA1	SNV	NS	c.3400G>T es11	p.Glu1134Ter	67.07	Unclassified
BRCA1	SNV	Splice site variant	c.135-1G>T es5	p.?	64.34	5
BRCA1	Deletion	Fs	c.5395delG es11	p.Ala1799HisfsTer6	59.29	5
BRCA1	Deletion	Splice site variant	c.22_212+1delAGG es4	p.?	19.33	4
BRCA1	Deletion	Fs	c.4964_4982delCT GGCCTGACCCCA GAAGA es16	p.Ser1655TyrfsTer16	69.65	5
BRCA1	Deletion	Fs	c.514delC	p.Gln172AsnfsTer62	46.33	5
BRCA1	Deletion	Fs	c.4964_4982del 5083del19	p.ser1655Tyrfs16	22.1	5
BRCA1	Deletion	Fs	c.4964_4982del 5083del19	p.ser1655Tyrfs16	65.4	5
BRCA1	SNV	M	c.4484G>T es14	p.Arg1495Met	81.98	5
BRCA2	Insertion	Fs	c.4284_4285insT es11	p.Gln1429SerfsTer9	92.23	5
BRCA2	Deletion	Fs	c.9377del es25	p.Gln3126Argfs*37	92.23	4
BRCA2	Insertion	Fs	c.4284_4285insT es11	p.Gln1429SerfsTer9	93.30	5
BRCA2	Deletion	Fs	c.6082_6086delGA AGA es11	p.Glu2028LysfsTer19	84.05	5
BRCA2	SNV	M	c.8419T es19	p.Ser2807Pro	17.88	3
BRCA2	Deletion	Fs	c.5851_5854delAG TT es 11	p.Ser1951TrpfsTer11	75.44	5
BRCA2	Insertion	NS	c.1842_1843insT	p.Asn615Ter	47.15	5
BRCA2	SNV	M	c.3049A>C	p.Ile1017Leu	46.1	3

*These PV/LPVs are present in the same proband showing double heterozygosity for BRCA1 and BRCA2 genes.
Abbreviations: PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; M= Missense; NS= Nonsense; VAF= Variant Allele Frequency.

Table 2 The PV/LPVs of BRCA1/2 genes in ovarian cancer patients tested for POLQ, ALDH1A1, FEN1, APEX2, FANCD2.

Gene	Type of variant	Molecular consequence	HGVS Nomenclature	Protein change	VAF (%)	Variant Class (PV/LPVs)
BRCA1	SNV	NS	c.1154G>A	p.W385Ter	67.2	5
BRCA1	SNV	M	c.5297 es21	p.Ile1766Ser	92.02	5
BRCA1	Deletion	Fs	c.3736delA es11	p.Thr1246ProfsTer18	79.78	5
BRCA1	Deletion	Fs	c.4964_4982del 5083del19	p.Ser1655TyrfsTer16	-	5
BRCA2	Insertion	Fs	c.4284_4285insT es11	p.Gln1429SerfsTer9	92.23	5
BRCA2	Deletion	Fs	c.9377del es25	p.Gln3126Argfs*37	92.23	4

*These PV/LPVs are present in the same proband showing double heterozygosity for BRCA1 and BRCA2 genes.
Abbreviations: PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; M= Missense; NS= Nonsense; VAF= Variant Allele Frequency.

Table 3 Baseline features and clinical-pathological information of OC patients.

Characteristics	BRCA1/2-Cohort	HRD positive No-BRCA Cohort	WT Cohort
	No. (%)	No. (%)	No. (%)
Number of patients	21 (29.2)	10 (13.9)	41 (56.9)
Age groups (y)			
≤40	1 (4.8)	/	3 (7.3)
41-50	2 (9.5)	/	7 (17.1)
51-60	7 (33.3)	1 (10)	11 (26.8)
>60	11 (52.4)	9 (90)	20 (48.8)
Surgery			
Yes	16 (76.2)	5 (50)	23 (56.1)
No	5 (23.8)	5 (50)	18 (43.9)
Bilateral			
Yes	3 (14.3)	2 (20)	4 (9.8)
No	18 (85.7)	8 (80)	37 (90.2)
Histological Subtype			
Serous low grade	1 (4.8)	/	24 (58.5)
Serous high grade	19 (90.5)	10 (100)	4 (9.8)
Endometrioid	1 (4.8)	/	6 (14.6)
Other	/	/	7 (17.1)
Histological Grade			
G1	1 (4.8)	/	1 (2.4)
G2	/	/	5 (12.2)
G3	20 (95.2)	10 (100)	29 (70.7)
Unknow	/	/	6 (14.6)
FIGO stage			
I-II	7 (33.3)	4 (40)	18 (43.9)
III	7 (33.3)	2 (20)	5 (12.2)
IV	1 (4.8)	2 (20)	/
Unknow	6 (28.6)	2 (20)	18 (43.9)
Metastatic at diagnosis			
Yes	1 (4.8)	2 (20)	/
No	20 (90.5)	8 (80)	41 (100)
Personal History of other tumors (HBOCS spectrum)			
Yes	3 (14.3)	1 (10)	/
No	18 (85.7)	9 (90)	41 (100)
Median Age at Diagnosis (y)			
Primary Tumor	61	69	59.5
Secondary Tumor	64	50	/
Time between 1st and 2nd Tumors (y)			
Median	15	50	/

Abbreviations: OC= Ovarian Cancer; WT= Wild-Type; y= years old.

Table 4 Baseline features and clinical-pathological information of OC patients, tested for POLQ, ALDH1A1, FEN1, APEX2, FANCD2.

Characteristics	BRCA1/2-Cohort	HRD positive No-BRCA Cohort	WT Cohort
	No. (%)	No. (%)	No. (%)
Number of patients	6 (31.6)	3 (15.8)	10 (52.6)
Age groups (y)			
≤40	/	/	1 (10)
41-50	1 (16.7)	/	2 (20)
51-60	4 (66.7)	/	3 (30)
≥60	1 (16.7)	3 (100)	4 (40)
Surgery			
Yes	4 (66.7)	1 (33.3)	6 (60)
No	2 (33.3)	2 (66.7)	4 (40)
Bilateral			
Yes	1 (16.7)	1 (33.3)	/
No	5 (83.3)	2 (66.7)	10 (100)
Histological Subtype			
Serous low grade	/	/	3 (30)
Serous high grade	5 (83.3)	3 (100)	5 (50)
Endometrioid	1 (16.7)	/	2 (20)
Histological Grade			
G1	/	/	/
G2	/	/	1 (10)
G3	6 (100)	3 (100)	8 (80)
Unknow	/	/	1 (10)
FIGO stage			
I-II	2 (33.3)	1 (33.3)	6 (60)
III	3 (50)	1 (33.3)	/
IV	/	1 (33.3)	/
Unknow	1 (16.7)	/	4 /40)
Metastatic at diagnosis			
Yes	/	1 (33.3)	/
No	6 (100)	2 (66.7)	10 (100)
Personal History of other tumors (HBOCS spectrum)			
Yes	2 (33.3)	/	/
No	4 (66.7)	3 (100)	10 (100)
Median Age at Diagnosis (y)			
Primary Tumor	58	65	56
Secondary Tumor	39.5	/	/
Time between 1st and 2nd Tumors (y)			
Median	15	/	/

Abbreviations: OC= Ovarian Cancer; WT= Wild-Type; y= years old.

Table 5 Median and Mean of POLQ gene expression.

POLQ	Mutated Cohort	Wild Type Cohort	Mutated + WT Cohorts
Median	0 [0 – 2,31]	0 [0 – 1,478]	0 [0 – 1,995]
Mean	1,138	2,81	2,017

Table 6 Median and Mean of APEX2 gene expression.

APEX2	Mutated Cohort	Wild Type Cohort	Mutated + WT Cohorts
Median	2,86 [0 – 3,88]	8,13 [2,5 – 12,44]	3,30 [0,47 – 11,55]
Mean	4,194	9,36	6,914

Table 7 Median and Mean of ALDH1A1 gene expression.

ALDH1A1	Mutated Cohort	Wild Type Cohort	Mutated + WT Cohorts
Median	5,23 [2,86– 135]	11,91 [5,26 – 14,58]	9,98 [3,165 – 35,35]
Mean	119,56	46,25	80,977

Table 8 Median and Mean of FANCD2 gene expression.

FANCD2	Mutated Cohort	Wild Type Cohort	Mutated + WT Cohorts
Median	0 [0 – 8,97]	13,35 [3,2– 46,49]	8,97 [0 – 34,76]
Mean	12,82	28,27	20,95

Table 9 Median and Mean of FEN1 gene expression.

FEN1	Mutated Cohort	Wild Type Cohort	Mutated + WT Cohorts
Median	11,68 [0 – 19,23]	18,1 [3,9 – 33,31]	16,79 [0 – 26,6]
Mean	14,78	22,74	18,97

Table 10 Median and Mean of POLQ gene expression in subgroup analysis of the wild type cohort divided by histotype.

POLQ	Low Grade Serous	High Grade Serous	Endometrioid	Wild Type Cohort
Median	0,87	0,938	0	0 [0 – 1,478]
Mean	5,2	0,938	0	2,81

Table 11 Median and Mean of APEX2 gene expression in subgroup analysis of the wild type cohort divided by histotype.

APEX2	Low Grade Serous	High Grade Serous	Endometrioid	Wild Type Cohort
Median	9,23	2,81	23,225	8,13 [2,5 – 12,44]
Mean	8,04	4,6	23,225	9,36

Table 12 Median and Mean of ALDH1A1 gene expression in subgroup analysis of the wild type cohort divided by histotype.

ALDH1A1	Low Grade Serous	High Grade Serous	Endometrioid	Wild Type Cohort
Median	9,98	13,83	186	11,91 [5,26 – 14,58]
Mean	9,41	12,45	186	46,25

Table 13 Median and Mean of FANCD2 gene expression in subgroup analysis of the wild type cohort divided by histotype.

FANCD2	Low Grade Serous	High Grade Serous	Endometrioid	Wild Type Cohort
Median	0	13,58	20,99	13,35 [3,2– 46,49]
Mean	27,33	31,72	20,99	28,27

Table 14 Median and Mean of FEN1 gene expression in subgroup analysis of the wild type cohort divided by histotype.

FEN1	Low Grade Serous	High Grade Serous	Endometrioid	Wild Type Cohort
Median	19,37	15,6	45,315	18,1 [3,9 – 33,31]
Mean	17,81	16,67	45,315	22,74

Figure 1 A) Homologous Recombination Repair (HRR): The mechanism of DNA double strand break repair by HRR. **B) Homologous Recombination Deficiency (HRD):** Representative cause-and-effect scheme, with reference to the main tests for HRD detection (Myriad MyChoicer HRD assay for GIS-score + BRCA1/2 mutation and Foundation Medicine NGS assay for LOH-score + BRCA1/2 mutation).

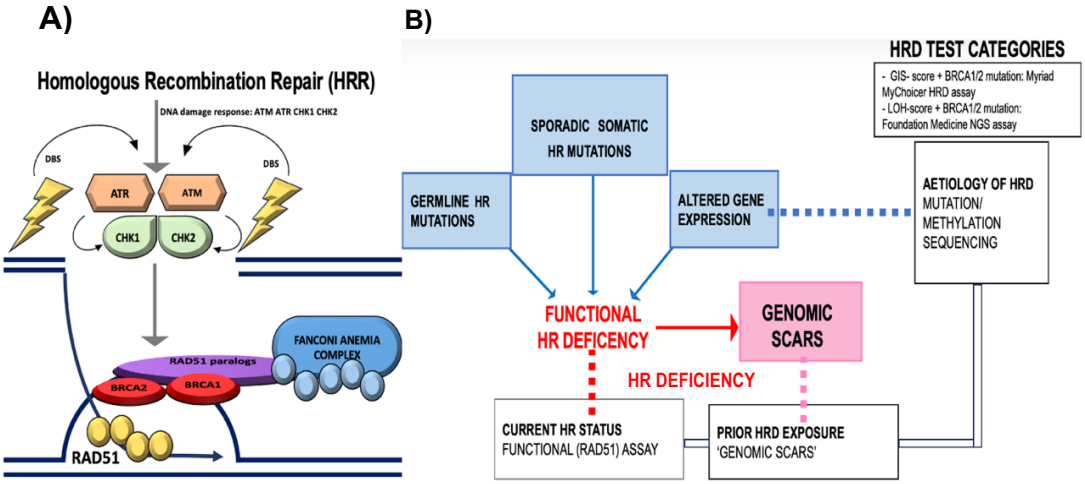


Figure 2 Polymerase Theta-Mediated End Joining (TMEJ): The mechanism of DNA double strand break repair by TMEJ in HR-deficient cells. Image reproduced by Barszczewska-Pietraszek G et al, 2022, Int J Mol Sci [89].

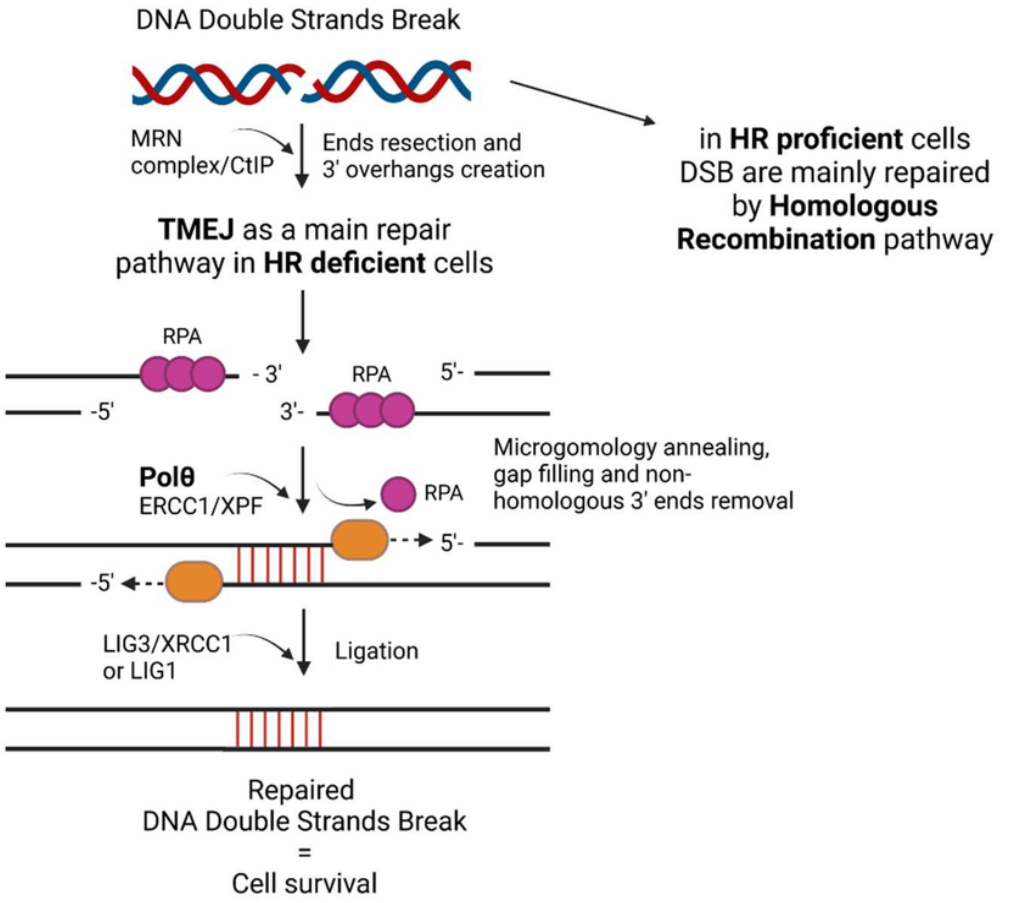


Figure 3 POLQ expression level in mutated cohort and in wild type cohort. Relative expression levels are represented for each group.

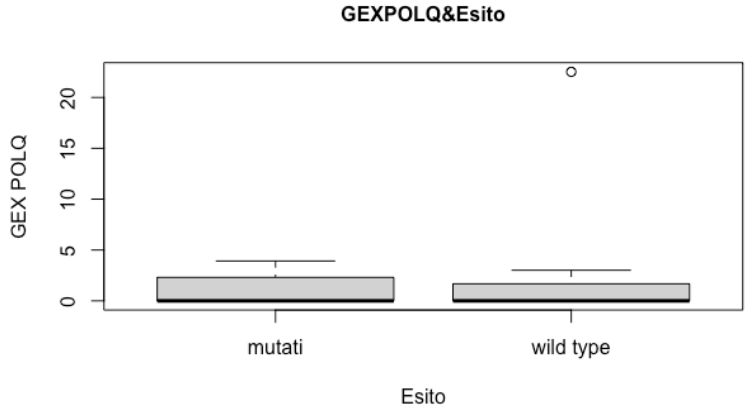


Figure 4 APEX2 expression level in mutated cohort and in wild type cohort. Relative expression levels are represented for each group.

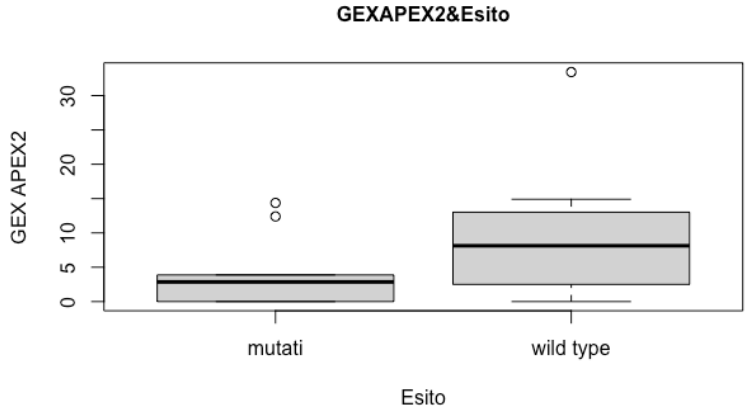


Figure 5 ALDH1A1 expression level in mutated cohort and in wild type cohort. Relative expression levels are represented for each group.

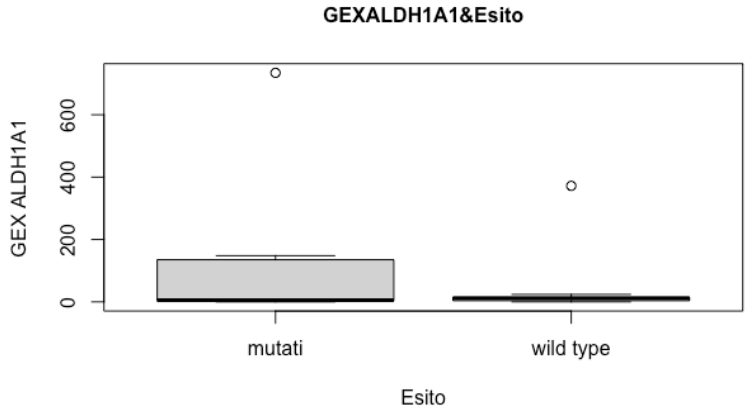


Figure 6 FANCD2 expression level in mutated cohort and in wild type cohort. Relative expression levels are represented for each group.

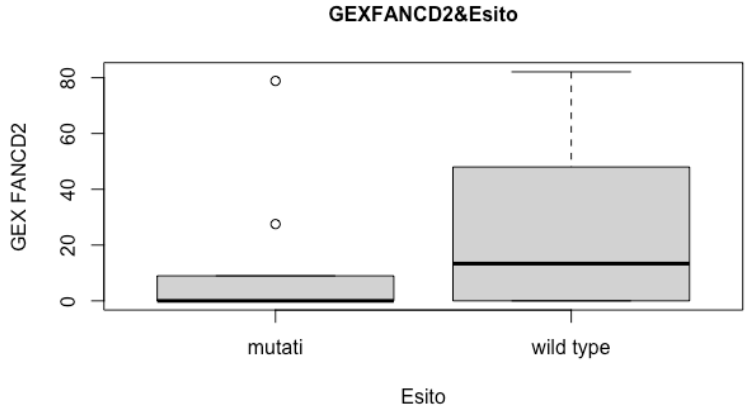


Figure 7 FEN1 expression level in mutated cohort and in wild type cohort. Relative expression levels are represented for each group.

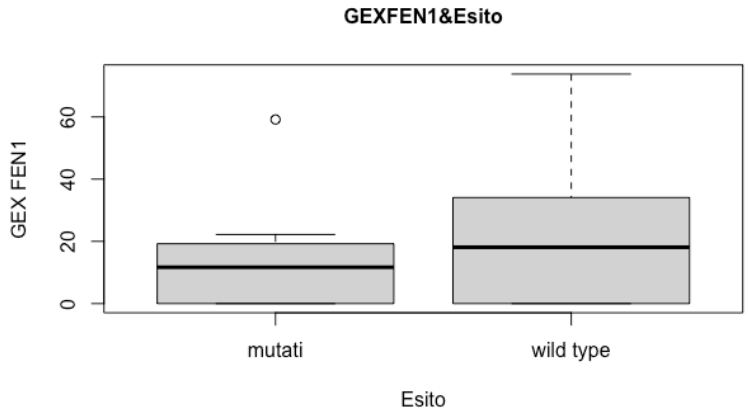
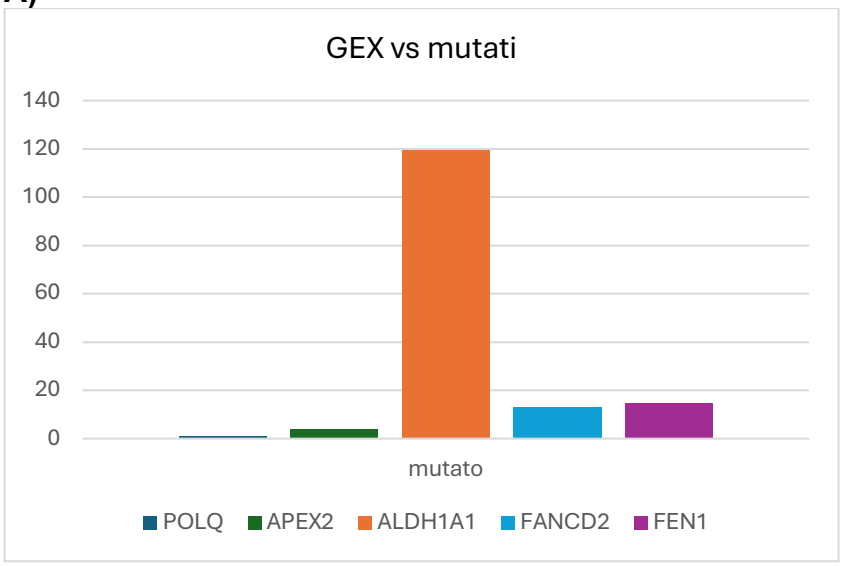
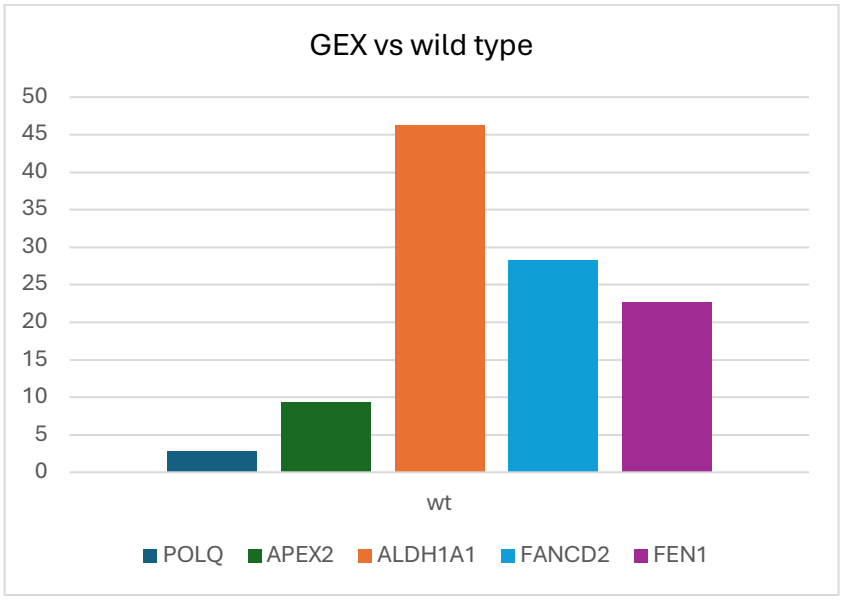


Figure 8 A) Graphic representation of the expression of POLQ, APEX2, ALDH1A1, FANCD2 and FEN1 in the mutated cohort. B) Graphic representation of the expression of POLQ, APEX2, ALDH1A1, FANCD2 and FEN1 in the WT cohort.

A)



B)



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Abstract

1. Real life use of biomarkers of homologous recombination deficiency (HRD) status beyond BRCA to predict the effectiveness of PARP inhibitors in ovarian cancer patients Poster Session “Prevention, Risk Reduction, and Hereditary Cancer”. Lorena Incorvaia, Chiara Brando, Alessandro Perez, Marco Bono, Daniela Cancelliere, Alessia Pivetti, Nadia Barraco, Silvia Contino, Chiara Annamaria Raso, Anna Paola Carreca, Valerio Gristina, Antonio Galvano, Tancredi Didier Bazan Russo, Luisa Castellana, Lavinia Insalaco, Daniele Fanale, Claudia Marchetti, Viviana Bazan, Ignazio Carreca, Antonio Russo 2023 ASCO Annual Meeting, Chicago, IL, United

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2. Pregnancy and miscarriage before epithelial ovarian cancer (EOC) diagnosis in patients carrying germline BRCA1/BRCA2 pathogenic variants. Tancredi Didier Bazan Russo, Mattia Puglisi, Elga Adriana Cipolla, Giovanni Colletta, Karen Carobene, Pietro Ferraro, Ugo Randazzo, Nadia Barraco, Silvia Contino, Anna Paola Carreca, Adriana Giusi Lo Bosco, Valerio Gristina, Antonio Galvano, Daniele Fanale, Alessandro Perez, Giuseppe Badalamenti, Viviana Bazan, Ignazio Ugo Carreca, Antonio Russo, Lorena Incorvaia; University of Palermo, Palermo, Italy; Ri.MED Foundation, Proteomic's Unit, Department of Research IRCCS ISMETT, Palermo, Italy; Department of Precision Medicine in Medical, Surgical and Critical Care, Palermo, Italy; University of Palermo, Palermo, Sicilia, Italy; Department of Biomedicine, Neurosciences and Advanced Diagnostics-BINRED, University of Palermo, Palermo, Italy; University of Palermo, Palermo, PA, Italy
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